

A Study of the Influence of Sperm Surface Proteins on the
Activity of Avian Spermatozoa In-vitro and In-vivo

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I certify that this thesis is the true and accurate
version of the thesis approved by the examiners.

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Abstract

Chicken spermatozoa undergo a post-testicular maturation process similar to that reported for mammals. Association of secreted epididymal proteins and glycoproteins with the sperm surface appears to be complete at the anterior ductus deferens. However, antigenic change to, and glycosylation and deglycosylation of seminal plasma and sperm surface-associated proteins, selective loss of proteins from the sperm surface, as well as secretion of some seminal plasma proteins and selective absorption of others by epithelial cells lining the excurrent ducts, continues in the ductus deferens and ampulla.

Hypertonic treatment or neuraminidase treatment of spermatozoa without apparent loss of sperm integrity in-vitro, reduced the ability of spermatozoa to gain access to the uterovaginal junction sperm storage tubules in-vivo following intravaginal insemination. This indicates a clear role for sperm surface-associated proteins and sperm surface sialic acid in vaginal sperm transport.

Sperm surface-associated proteins were also extracted by glycerol in a temperature-dependent and concentration-dependent manner. This was accompanied at room temperature by a glycerol concentration-dependent reduction in sperm motility. These effects may be implicated in the contraceptive action of glycerol in the chicken vagina.

Spermatozoa from several avian and mammalian species entered quail uterovaginal junction SSTs in-vitro, and turkey spermatozoa were found in chicken uterovaginal junction SSTs following uterovaginal junction but not intravaginal insemination, thus showing that the SSTs are not selective, and identifying the vagina as a major site of oviducal sperm selection.

Spermatozoa washed from the vagina following intravaginal insemination had immunoglobulin bound to their surface, which was shown to be associated with cell death. Spermatozoa recovered from the anterior oviduct however, were generally devoid of bound immunoglobulin. Furthermore, sperm access to the newly ovulated egg in-vivo following incubation with vaginal mucosa in-vitro did not differ significantly from that of control spermatozoa despite fewer viable spermatozoa inseminated, suggesting a true 'selection' of spermatozoa in the vagina.

Ovarian pocket fluid, postulated to be the milieu in which fertilisation takes place in-vivo, altered sperm surface

antigenicity and reduced sperm motility in-vitro, suggesting that spermatozoa may naturally undergo oviduct-induced changes prior to fertilisation.

Sperm-egg interaction appears specific and receptor-mediated, as in mammals. Spermatozoa showed no preference for the animal pole of the egg, and heterologous gamete combinations indicated limited, order-dependent rather than species-dependent specificity.

Presenting carbohydrate residues on the sperm surface and sperm surface antigenicity showed a lack of change proportional to species divergence, although the vagina clearly presents the main barrier to interspecies fertilisation within the order Galliformes.

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CHAPTER 1. INTRODUCTION

1. INTRODUCTION

1.1. General Introduction

Considerable efforts have been made over the past 30 years to elucidate the mechanisms involved in post-testicular sperm maturation, sperm-oviducal interaction and sperm-egg interaction in mammals. In addition to the scientific value of the large volume of information generated by such work, some aspects have subsequently found practical application in medicine and agriculture.

Assisted conception, both in the human and mammalian fields, is currently a growth industry. In-vitro fertilisation (IVF) is a recommended and established treatment for infertility resulting from blocked fallopian tubes, and in recent years has found application in other categories of subfertility (see Lee, 1991). Furthermore, viable offspring have been produced from the transfer of animal embryos produced by in-vitro maturation (IVM) and IVF, to recipient animals (Xu et al., 1987; Goto et al., 1987). Such techniques may find application in the production of embryos from valuable but infertile livestock (see Fayrer-Hosken & Caudle, 1990). IVF also finds application in the production of transgenic farm animals (Hammer et al., 1985) and is a valuable technique in investigations of the physiology of gamete maturation, interaction and fertilisation.

Other techniques applied to the field of human infertility which derive their origins from research into sperm-oviducal interaction and sperm-egg interaction include gamete intrafallopian transfer (GIFT), intrauterine insemination (IUI), direct intraperitoneal insemination (DIPI) and microinjection of spermatozoa into oocytes (see Lee, 1991). Some of these techniques e.g. GIFT, may also find application in livestock production (see Fayrer-Hosken & Caudle, 1990).

In domestic birds, reduction in the fertility of a breeding flock is not uncommon, especially in broiler breeders over 50 weeks old, which may produce only 70% fertile eggs. The normal solution to such reduced fertility is to increase the male to female ratio or resort to artificial insemination. However, such a strategy does not solve the underlying cause and encourages the production of birds with inferior reproductive capabilities.

In the U.K., the annual production of broiler chickens has a total value of £600M, with the value of turkey production being around £200M (M.A.F.F. Bulletin, 1990). Therefore, even a 1% improvement in the normal mean fertility level of 87-90% would be worth more than £8M per annum.

The first logical step towards improving reproductive capability is the elucidation of physiological mechanisms

involved. Although much work has been carried out on egg production and to a lesser extent spermatogenesis, little is presently known about post-testicular sperm maturation, oviducal sperm transport and storage, and sperm-egg interaction in domestic birds.

The work detailed in this thesis has been concerned with the characterisation of sperm surface changes occurring during transport through the excurrent duct system of the chicken, and subsequently the oviduct; investigation of the role of sperm surface associated proteins in oviducal transport of spermatozoa; investigation of sperm-egg interaction; and the elucidation of the relative importance of the vagina and inner perivitelline layer of the egg as barriers to interspecies fertilisation involving closely and distantly related avian species.

The work undertaken has been comprehensive in relation to the stages of the sperm life cycle, dealing with spermatozoa in the male reproductive tract and throughout the female reproductive tract to the point of fertilisation. The introduction to the thesis has thus been arranged in a sequential manner to provide relevant background information on areas studied, dealing initially with male reproductive tract anatomy, post-testicular maturation and semen composition, and proceeding via female reproductive tract anatomy and oviducal sperm transport, selection and storage, to sections covering

sperm capacitation, sperm-egg interaction and interspecies fertilisation.

Most information currently available on the areas studied in this project has been obtained from mammalian research, making mammals the logical vertebrate class to draw comparisons from. Accordingly, information on mammalian reproductive mechanisms has generally been provided alongside that for domestic birds.

1. 2. Sperm Maturation

1. 2. 1. Testicular Sperm Maturation in Domestic Birds

1. 2. 1. 1. Anatomy of the Chicken Testis

The testes are found dorsally in the body cavity at the cranial pole of the kidneys, and measure approximately 30-60 mm in length and 25-30 mm in diameter in sexually mature males.

The non-septate testicular parenchyma is enveloped by a thin, highly vascularised tunica albuginea, which in turn underlies a serous membrane. The seminiferous tubules form a complex anastomosing network (Lake, 1957), their outer wall comprising a thin layer of connective tissue containing contractile myoid cells (Rothwell & Tingari, 1973), and their epithelium comprising Sertoli cells and germinal cells at various stages of development (Lake, 1981). The cell body of the Sertoli cell is situated at the base of the seminiferous tubule epithelium, with fine projections of cytoplasm between germinal cells (Cooksey & Rothwell, 1973). Sertoli cells play a supportive and nutritive role towards developing spermatogenic cells, phagocytise degenerating spermatogenic cells, and by virtue of tight intercellular junctions, form the blood-testis barrier (Tortora and Anagnostakos, 1990). They are also believed to possess steroidogenic activity (Siller, 1956). The interstices between seminiferous

tubules comprise loose connective tissue, Leydig cells which secrete androgens in response to stimulation by luteinising hormone (see Lake, 1984), and vascular tissue.

The testicular arteries arise from the cranial renal arteries and are responsible for organ perfusion. These enter the epididymal border of the testes, forming anastomosing arterial arcades between the seminiferous tubules. Venous return is via short testicular veins which flow into the caudal vena cava.

1. 2. 1. 2. Spermatogenesis

The seminiferous tubules of the testes are lined with spermatogonia, which display mitotic activity. Groups of filial cells extend into the tubule lumen, differentiating at each mitotic division, and finally forming primary spermatocytes, which subsequently undergo meiotic cell division. The prophase of the first meiotic division is elongated, being 5.5 days in chickens (see Reviers, 1975) and comprises leptotene, zygotene, pachytene, diplotene and diakinesis stages. The two resultant secondary spermatocytes undergo a further division to produce four haploid spermatids, which subsequently undergo spermiogenesis, lasting approximately 7 days in chickens (see Reviers, 1975). Spermiogenesis is the modelling process which ultimately results in the morphologically mature spermatozoon (Findlay, 1984) and involves

flagellar, midpiece and acrosome development, in addition to condensation of the nucleus. The total duration of spermatogenesis in the chicken is approximately 12 days (see Reviers, 1975).

1.2.2. Post-testicular Sperm Maturation

1.2.2.1. Anatomy of the Excurrent Duct System of the Testis

The excurrent duct system of the testis (Figure 1) comprises a short epididymal region, approximately 20 mm in length, and the ductus deferens, which is approximately 140 mm in length (Tingari, 1971). The epididymal region comprises the rete testis, ductuli efferentes, connecting ductules, and ductus epididymidis (Lake, 1957). The whole duct complex immediately adjacent to the testis is embedded in a fibrous stroma containing a few muscle fibres.

The rete testis comprises a complex of thin walled irregular channels surrounded by connective tissue, lined by simple squamous to low cuboidal epithelial cells, which communicates with the seminiferous tubules via short straight tubules in which no spermatogenesis occurs (Tingari, 1971).

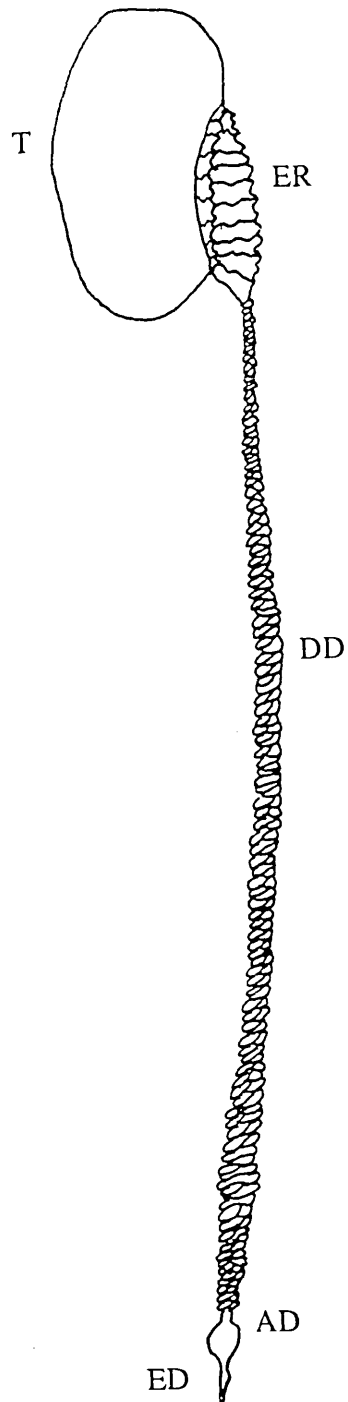


Figure 1. Diagrammatic representation of the reproductive tract of the male chicken: T, testis; ER, epididymal region (comprising rete testis, connecting ductules and ductus epididymidis); DD, ductus deferens; AD, ampulla of ductus deferens; ED, ejaculatory duct which protrudes into the cloaca.

The ductuli efferentes are large tubules connecting the rete testis and the connecting ductules, and are lined with non-ciliated and ciliated columnar cells, found alternating individually or in groups. The connecting ductules, which are considerably narrower, are lined predominantly with ciliated columnar cells, although non-ciliated cells are evident. The ductules widen prior to connection with the ductus epididymidis, and the epithelial lining becomes similar to that of the ductus epididymidis (Tingari, 1971, 1972).

The ductus epididymidis is a convoluted tube lined with non-ciliated columnar cells, which communicates with the histologically similar ductus deferens (Tingari, 1971). The ductus deferens runs parallel to the ureter, from the cauda epididymis to the cloacal urodeum. It is a highly coiled structure and expands into receptacles which penetrate the cloacal wall and terminate in short papillae which enter the urodeal cavity. The wall of the ductus deferens is fibromuscular, and spermatozoa are transported by fluid flow and peristaltic movements of the muscle fibres (Reviers, 1975).

The mammalian epididymis is considerably longer than that of domestic birds, being approximately 20 m long in man, and 40-60 m long in the bull, boar and stallion (see Jones, 1989), and is divided into three anatomical regions; caput (head), corpus (body) and cauda (tail). In most mammalian species the duration of sperm transport

through the epididymis is between 8 and 12 days, and it is well established that further maturational processes take place here.

The ductus deferens of the chicken is regarded by some as equivalent to the corpus and caudal regions of the mammalian epididymis (Tingari, 1971, 1972; Lake, 1981), the juxtatesticular epididymal region corresponding with the mammalian caput epididymidis. Evidence for such a claim includes: changes in the luminal diameter between the cranial aspect of the epididymal duct and the caudal aspect of the ductus deferens, similar to those observed in some mammals; similarity of the epithelium lining the ductus deferens of the chicken to that lining the corpus and cauda epididymidis of the rat and mouse; the observation that cell types found in the chicken epididymal duct and ductus deferens epithelia are identical; and histological evidence for apocrine secretory activity in the ductus deferens (see Tingari, 1971).

Following spermatogenesis and spermiogenesis, avian spermatozoa are released via the rete testis into the ductuli efferentes, which drain into the ductus epididymidis, and then into the ductus deferens. In the chicken, transport of spermatozoa from the testes to the caudal region of the ductus deferens takes between 1 and 4 days, depending on the frequency of ejaculation (Reviers, 1975).

The luminal contents vary in different areas of the duct system, with the lowest and highest sperm densities observed in the rete testis and initial narrow region of the connecting ductules, respectively. Spermatozoa are fairly uniformly distributed throughout the other areas of the tract (Tingari, 1971).

1.2.2.2. Mammalian Post-testicular Sperm Maturation

Mammalian testicular spermatozoa are immotile and infertile (Mann & Lutwak-Mann, 1981), development of progressive motility and fertilising ability depending on modifications occurring during sperm transport through the epididymis (Bedford, 1979; Dacheux & Paguignon, 1980; Olson, 1984; Cooper, 1986). Some changes are intrinsic to spermatozoa (Myles et al., 1987), others occurring as a result of interaction with epididymal fluid.

Modifications to the sperm surface may result from the regional surface acquisition of specific protein and/or glycoprotein (Olson & Hamilton, 1978; Feuchter et al., 1981; Jones et al., 1983; Russell et al., 1984), enzymic modification e.g. by glycosidases, glycosyltransferases or proteases, of existing sperm surface molecules (Hamilton, 1980; Cossu & Boitani, 1984), or the masking or unmasking of pre-existing molecules or epitopes (Olson & Danzo, 1981; Eddy et al., 1985).

Concurrent with sperm surface modification are changes in lectin binding patterns (Nicolson et al., 1977; Olson & Danzo, 1981; Srivastava & Olson, 1991) and an increase in net negative surface charge (Yanagimachi et al., 1972; Moore, 1979) due mainly to sialic acid residues (see Toshimori et al., 1991) secreted by the epididymal epithelium as the terminal sugar of sialoglycoproteins which bind to the sperm surface (Acott & Hoskins, 1981; Rifkin & Olson, 1985; Feuchter et al., 1988). It has also been established that phospholipid, cholesterol and bulk phospholipid-bound fatty acids of whole spermatozoa and sperm plasma membrane undergo change during epididymal transit, accompanied by plasma membrane fluidity changes (see Hall et al., 1991).

Other reported biochemical and morphological changes accompanying epididymal transit include size, shape and internal structure of the acrosome, cohesiveness between the outer acrosomal membrane and overlying sperm plasma membrane, light scattering properties, lipoprotein content, electrophoretic mobility, and loss of the cytoplasmic droplet from the tail (see Nicolson et al., 1977). Furthermore, maturation-related changes in elemental concentrations around or on the sperm surface have been reported (see Ashizawa et al., 1988).

Epididymal transit is accompanied by functional maturation in mammals. The caput epididymidis is the most active site of androgen-controlled protein synthesis and

secretion (Tezon et al., 1985), and the ability of spermatozoa to bind specifically to the zona pellucida is developed here. However, progressive motility and the ability to penetrate and fertilise the oocyte are developed in the corpus epididymis, with the ability to initiate normal embryonic development arising in the cauda epididymidis (Fournier-Delpech et al., 1983; Fournier-Delpech & Courot, 1987). Some epididymal fluid components which associate with the sperm surface have had their physiological role identified e.g. forward motility protein (Acott & Hoskins, 1981), acrosome stabilising factor (Eng & Oliphant, 1978), and glycoproteins implicated in sperm-zona pellucida binding (Leyton & Saling, 1989 a,b).

1. 2. 2. 3. Avian Post-testicular Sperm Maturation

Like their mammalian counterparts, avian spermatozoa are known to undergo a further maturational process during their transport through the excurrent duct system. Esponda and Bedford (1985) observed that epididymal and ductus deferens fluid components associate with the chicken sperm surface. In common with mammals, migration and eventual shedding of the cytoplasmic droplet from the tail midpiece (Bedford, 1979), and changes in elemental concentrations around or on the chicken sperm surface (Ashizawa et al., 1988) are observed during sperm transit through the excurrent duct system of the chicken.

In contrast to mammalian epididymal spermatozoa, which suffer a precipitous loss of viability following removal of testicular androgen (Bedford, 1979), chicken spermatozoa in the excurrent duct system are not influenced by testicular androgens (Munro, 1938a). Furthermore, the failure of cationised colloidal ferric oxide and FITC-conjugated lectins to indicate changes in anionic groups and specific oligosaccharide residues respectively, led Bedford (1979) to conclude that macromolecules acquired by chicken spermatozoa during epididymal and ductus deferens transit were not glycoprotein. This hypothesis has more recently received support from Esponda and Bedford (1985) who identified only one glycoprotein band in chicken seminal plasma, following protein separation on isoelectric focussing gels.

In contrast to the mammalian case, few studies have been carried out on the extragonadal maturation of spermatozoa in domestic birds. As a result, little is known about the surface and non-surface changes experienced by avian spermatozoa during transit of the epididymis and ductus deferens (see Bedford, 1979; Esponda & Bedford, 1985; Morris et al., 1987), and specific functions have not yet been attributed to individual sperm surface associated components. However, it is clear that there are a number of fundamental differences between the functions of avian and mammalian sperm surface associated components. It has been established for example, that extra

gonadally-adsorbed avian sperm surface-associated components are not involved in recognition and sperm binding to the inner perivitelline layer of the egg, generation of the acrosome reaction, or binding to the oolema and initiation of gametic fusion, since testicular spermatozoa, which lack exposure to epididymal or ductus deferens secretory components, can fertilise ova if inseminated intramagnally (Howarth, 1983). In contrast to the mammalian case, some chicken spermatozoa are fully motile in the testes (Bedford, 1979; Ashizawa & Sano, 1990), so the acquisition of a forward motility protein during post-testicular maturation would seem unnecessary.

The observation that testicular spermatozoa inseminated intravaginally do not fertilise ova (Munro, 1938b, Howarth, 1983) suggests a possible role for sperm surface-associated components in oviducal sperm transport and storage. Furthermore, sperm transport from the uterus to the infundibulum is passive (Allen & Grigg, 1957), suggesting that sperm surface associated components might exert their influence at the vagina and/or uterovaginal junction.

1. 2. 3. Avian Semen

1. 2. 3. 1. Seminal Plasma

Semen is collected by massaging the back of the male, eliciting the erection of the phallic structures (median and 2 lateral phallic bodies found on the ventral lip of the cloacal vent) and papillae. Erection of these structures is by lymphatic engorgement, lymph being formed by the paracloacal vascular bodies situated lateral to the receptacles of the ductus deferens. The semen is then squeezed into a collecting vessel (Lake & Stewart, 1978; Lake, 1981).

There are no accessory genital gland secretions, but a lymph-like transparent fluid can be expressed from lymphatic folds on the proctodeal floor during semen collection (Nishiyama & Ogawa, 1961).

Seminal plasma is produced as a result of secretion by the epididymis and ductus deferens (Esponda & Bedford, 1985) and absorption by the epithelial cells lining the excurrent duct system (Nakai et al., 1989). It is a complex medium with a total protein content of 8 mg/ml (Lake, 1966), including glycoprotein, cholinesterases, acid and alkaline phosphomonoesterases (Bell & Lake, 1962), oxidative and hydrolytic enzymes (McIndoe & Lake, 1974) and proteinase inhibitor. Several amino acids are found free in seminal plasma, with the glutamic acid

concentration considerably elevated compared with that in blood plasma (Lake & Hatton, 1968), and steroids may be present (Budras & Sauer, 1975). Other known constituents include lactic acid, pyruvic acid, α -ketoglutarate, carnitine, acylcarnitine, creatine, glucose, chloride, sodium, potassium and magnesium, all, with the exception of sodium, at concentrations differing from their respective concentrations in blood plasma, (Lake & Wishart, 1984).

There are conflicting views on whether transparent fluid is a normal constituent of seminal plasma and what its function might be. Lake (1984) suggested that it might reduce the acidity on the surface of the erectile tissue in the cloaca to prevent damage to spermatozoa. However, Terada et al. (1981) have suggested that it may provide stimulation of sperm motility.

1. 2. 3. 2. The Spermatozoon

Chicken semen contains between 3×10^6 and 8×10^6 spermatozoa/ml (Lake & Stewart, 1978).

The chicken spermatozoon is of the sauropsid type, characteristic of non-passerine birds (Asa & Phillips, 1987). The nucleus is cylindrical, approximately $12.5 \mu\text{m}$ long and slightly bent, the acrosome being approximately $2 \mu\text{m}$ long and conical. The subacrosomal space which

houses the perforatorium, separates the acrosome from the nucleus. The acrosome contains proteolytic enzymes which digest a pathway for the penetrating spermatozoon through the inner perivitelline layer of the oocyte (Bakst & Howarth, 1977b; Okamura & Nishiyama, 1978; Koyanagi et al., 1988).

The tail is approximately 80 μm in length and comprises four regions: neck, midpiece, principal piece and end piece. The neck region contains the centriolar complex, comprising the proximal and distal centrioles. Here, the nucleus is joined to the midpiece by a non-striated connecting piece and proximal centriole. Approximately 30 mitochondria surround the distal centriole and apical aspect of the axoneme at the midpiece. The axoneme originates from the distal centriole as 9 pairs of triplet tubules which change to a configuration of 9 pairs of doublets, and continues to the end of the principal piece (Lake et al., 1968). The principal piece and midpiece are separated by an annulus (Lake et al., 1968). The principal piece is approximately 70 μm long and comprises a sheath of amorphous material (Lake et al., 1968) which surrounds the axoneme. The end piece is short and comprises the axoneme only. The entire spermatozoon is bounded by a plasma membrane.

Spermatozoa from different species of domestic birds appear morphologically similar. Turkey spermatozoa are of a similar overall length (75-80 μm) to those of the

chicken (Thurston & Hess, 1987), but possess an extended midpiece, with a shorter, less curved nucleus (Lake & Wishart, 1984). Quail spermatozoa have a considerably greater overall length (approximately 140 μ m; M.G. Steele, unpublished observation) with an extended nucleus and a longer and thicker flagellum.

1. 3. Sperm-Oviducal Interaction

1. 3. 1. Anatomy of the Avian Oviduct

In the chicken, as in most birds, only the left Mullerian duct develops into a functional oviduct (Bakst, 1987a). The oviduct is anatomically and functionally divided into five segments: the infundibulum, magnum, isthmus, uterus, and vagina (Gilbert, 1979) (Figure 2). Approximately 0.6 m in length, the oviduct has a mucosal lining enveloped by connective tissue containing blood vessels and nerves. Beyond this lie smooth muscle layers, bounded by a serosa.

The infundibulum is approximately 90 mm in length and is lined with ciliated epithelium, with scattered bundles of smooth muscle fibres in the connective tissue layer. The posterior infundibulum converges into a narrow walled tube lined with longitudinal ridges, comprising grooves of ciliated and secretory cells, and contains the the secondary sperm storage sites, the infundibular sperm storage tubules (Van Drimmelen, 1946; Fujii & Tamura, 1963). The duration of the passage of the ovum through the infundibulum is approximately 15 to 30 minutes (Warren & Scott, 1934), and fertilisation occurs here (Lake, 1984). The outer perivitelline layer and first layer of albumen are secreted by the posterior portion of the infundibulum (Howarth, 1974; Bakst & Howarth, 1977b).

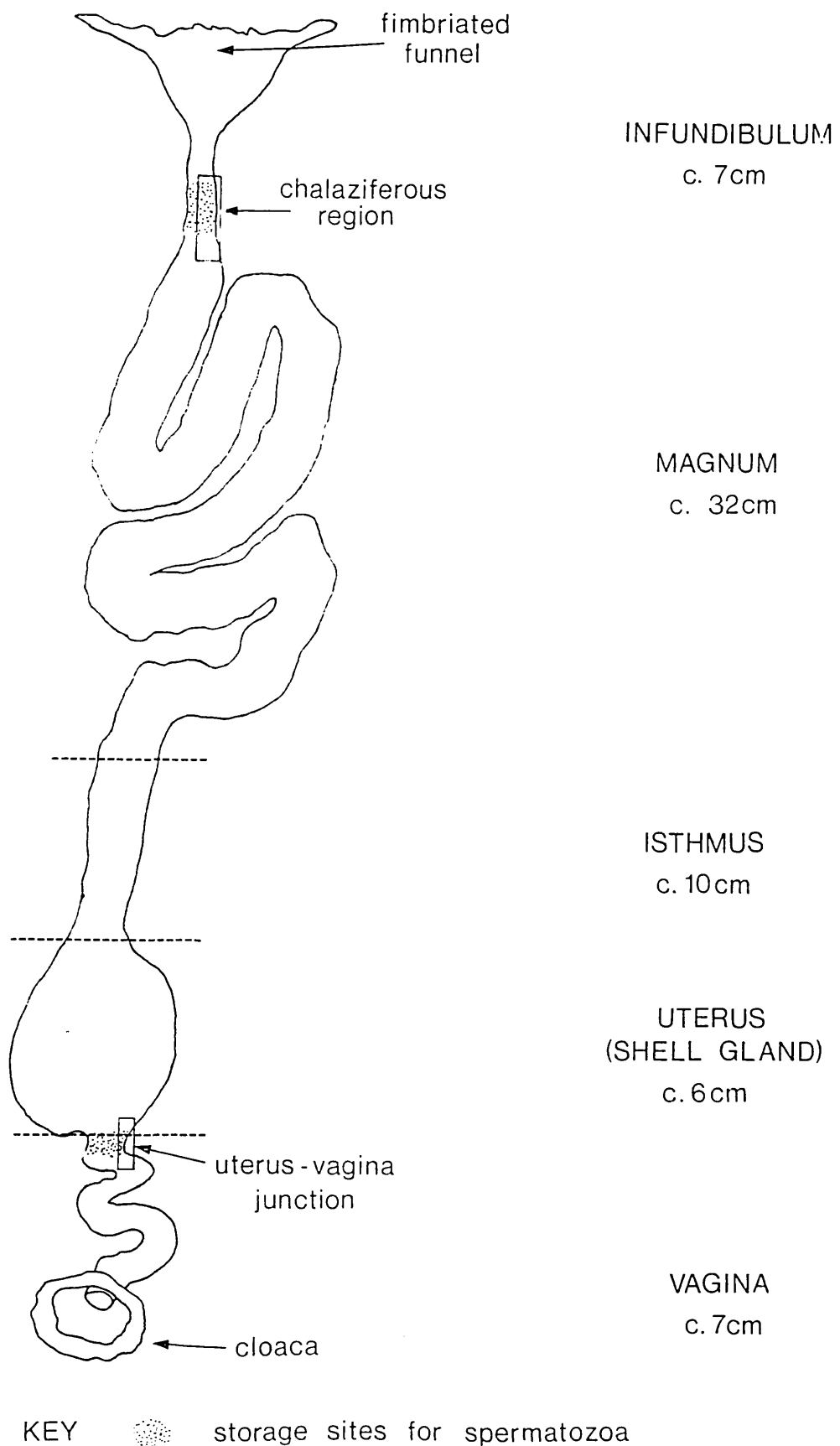


Figure 2. Diagrammatic representation of the reproductive tract of the female chicken.

The magnum is approximately 320 mm in length, with a thick mucosal layer and well developed muscular wall. The mucosa is ridged and lined with secretory epithelium containing well developed albumen-secreting tubular glands. Protein production and secretion are coordinated with egg formation, the duration of secretion being approximately 3 hours.

Between the magnum and isthmus is a narrow zone without glandular mucosa. The isthmus is approximately 100 mm long with a ridged mucosa comprising ciliated secretory cells, and containing tubular glands which secrete the shell membrane proteins. The egg remains in this region for approximately one hour.

The uterus is approximately 60 mm in length with a well developed musculature. The mucosa comprises a ciliated surface epithelium containing tubular glands and is organised in longitudinal folds which are intersected by transverse furrows, giving a leaf-like appearance. The egg remains in the uterus for approximately 20 hours, during which a calcified shell is formed.

Between the uterus and vagina lies the uterovaginal junction, which macroscopically takes the form of a sphincter, but in fact comprises a series of tortuous tissue convolutions (Bohr et al., 1965). Located within the uterovaginal junction are the primary sperm storage sites (Bohr et al., 1964a; Fujii & Tamura, 1963). These

take the form of blind ended tubules which are occasionally branched (Lake, 1967; Burke et al., 1972), and arise as invaginations of the uterovaginal mucosa, often from the base of mucosal crypts. The mucosa and crypts are lined with pseudostratified, ciliated columnar epithelium, the sperm storage tubules (SSTs) comprising a single layer of columnar cells ciliated at the proximal aspect, and non-ciliated deeper within the tubule. The fine structure of SST cells has been described in detail (Tingari & Lake, 1973; Bakst, 1987a).

The vagina is the most posterior segment of the reproductive tract and serves as a conduit between the uterus and the cloaca. It varies between 80 and 120 mm in length and has a muscular wall comprising an outer longitudinal layer and well developed circular layer. The mucosa comprises ciliated columnar cells.

1. 3. 1. 1. The Ovum

The megalecithal chicken ovum is approximately 33 mm in diameter at ovulation and comprises an animal pole and a vegetal pole. The animal pole appears as a disc approximately 4 mm in diameter on the surface of the ovum and contains the haploid nucleus, white yolk spheres and clear vacuoles. The surface of this region is bounded by a continuous plasma membrane from which microvilli project into the perivitelline space (Bakst & Howarth, 1977c;

Bakst, 1978). Bakst (1978) described 3 distinct regions of the germinal disc; the germinal centre, the periblastic region, and the outer periblastic region. The vegetal pole lacks organelles and the oolema is discontinuous, forming few microvillar projections.

The plasma membrane of the freshly ovulated ovum is surrounded by the inner perivitelline layer, which is produced by ovarian granulosa cells (Kido & Doi, 1988). This layer is 1-3.5 μm thick and comprises a three dimensional network of thick cylindrical fibres (Bellairs et al., 1963), the spaces between fibres being filled with a ground substance (Bakst & Howarth, 1977b). The thick fibres overlying the animal pole are thinner and more numerous than those overlying the vegetal pole (Perry et al., 1978). Kido et al. (1975) and Kido & Doi (1988) identified three major glycoproteins (GPI, GPII, GPIII) in the inner perivitelline layer. Burley and Vadehra (1989) have postulated that these glycoproteins may perform similar roles to mammalian zona pellucida proteins ZP1, ZP2 and ZP3.

The outer perivitelline layer varies between 3 and 8.5 μm in thickness and is in contact with the albumen (Bellairs et al., 1963; Jensen, 1969). This layer comprises a varying number of sublayers comprising a lattice-work of fine fibrils (Jensen, 1969) and is composed mainly of lysozyme, vitelline membrane outer I (VMOI), and ovomucin (see Burley & Vadehra, 1989).

Between the inner and outer perivitelline layers is a thin membrane approximately 50-100 nm thick. This takes the form of a undulating sheet-like structure and is known as the continuous membrane (Bellairs et al., 1963).

Bakst and Howarth (1977b) observed the barrier-like quality of the outer perivitelline layer with respect to sperm penetration, and suggested that this layer is either impenetrable by chicken sperm acrosin, or that ovomucin and other albumen proteins may inactivate acrosin, in a similar fashion to that observed for sialoproteins of Cowpers gland mucin and fetuin on capacitated rabbit spermatozoa.

1.3.1.2. Distribution of Immunoglobulin-Containing Cells Throughout the Oviduct

The following description provides anatomical background information relating to work carried out in this study to identify an immunological basis for oviducal sperm selection in domestic birds. The topic of sperm selection is covered in section 1.3.3..

The immunological status of the female chicken reproductive tract has received little attention, previous work having been concerned mainly with maternal transmission of passive immunity via the egg (See Kimijima et al., 1990).

Kimijima et al. (1990) studied the distribution of lymphoid tissues and immunoglobulin containing cells, and the localisation of immunoglobulins in the oviducal walls of laying chicken hens. Occasional accumulations of lymphocytes were identified, mainly in the middle infundibulum, isthmus, uterus and vagina.

The highest concentrations of immunoglobulin containing cells are found in the mucosal connective tissue of the magnum and isthmus, with IgG-containing cells predominating, although IgG, IgA and IgM are present in secretions throughout the oviduct (see Kimijima et al., 1990). However, IgA and IgG containing cells are found in comparable numbers in the vagina (Kimijima et al., 1990).

Immunoglobulins are also localised in superficial epithelial cells (SECs) and glandular cells throughout the oviduct. IgG containing SECs predominate, with large numbers of cells found in the infundibulum, isthmus and uterus.

Although high numbers of IgG, IgA and IgM containing glandular cells found in the magnum are believed to be mainly involved in the transfer of immunity to the egg, other immunoglobulin containing cells throughout the oviduct are believed to play a role in local defence of mucosal surfaces (Kimijima et al., 1990).

Previous work concentrating on the local immune status of the vagina and uterovaginal junction in domestic birds has been carried out in attempts to determine the cause of seasonal decline in fertility, a phenomenon peculiar to turkeys, in which hen fertility declines over the course of the egg production season.

Kirk et al. (1989) observed lymphocytes between contiguous cells of the uterovaginal junction SSTs and within SST lumen from fertile and infertile turkey hens, and Bakst (1987b) reported that some luminal lymphocytes contain cytoplasmic IgG. Schuppin et al. (1984) have postulated that SST luminal lymphocytes may be suppressor T cells which function to prevent an immune response against spermatozoa.

Kirk et al. (1989) reported the presence of IgG-containing cells near the basal lamina of the vaginal epithelium and the presence of IgG on the epithelial surface.

1. 3. 2. Oviducal Sperm Transport

1. 3. 2. 1. Mammals

In mammals, semen is deposited either in the vagina around the external cervical os e.g. rabbit, cow, sheep, goat and man, or into the uterine cavity e.g. pig, horse, dog, rat and mouse (Mortimer, 1978).

Vaginal deposition is followed almost immediately by the penetration of cervical mucus by motile spermatozoa, a process that continues over a period of several hours (Overstreet et al., 1989). Spermatozoa are believed to progress using their own motility (Overstreet & Katz, 1990) towards the cervical mucosa in the direction of alignment of the mucin glycoprotein molecules (Mattner, 1966), and spermatozoa are subsequently found associated with the cervical mucosa (Edey et al., 1975). Seminal plasma is not transported from the vagina to the uterus (Asch et al., 1977), indicating the importance of sperm motility in penetration and migration through the cervix.

It has been suggested that the cervix might have a function in sperm selection (Overstreet, 1983; Freundl et al., 1988; Katz et al., 1989; Cohen & Gregson, 1978; Overstreet et al., 1989) and may act as a sperm reservoir (Mattner, 1968; Zinaman et al., 1989). Furthermore, there is evidence for the initiation of capacitation in the cervix (Overstreet & Katz, 1990). However, in the

rabbit, which has little cervical mucus, the cervix may be primarily involved in sperm selection, with the sperm reservoir in the vagina (see Overstreet et al., 1989).

For species in which semen is deposited in the uterus, sperm selection and storage may occur at the uterine glands and uterotubal junction (Overstreet et al., 1989). However, in most species the uterus is believed to serve as a conduit through which spermatozoa, at their most active in the entire reproductive tract (Cooper et al., 1979), can access the oviduct. Spermatozoa may require vigorous flagellar activity to pass through the uterotubal junction, which may be the primary site of sperm selection in species in which semen is deposited in the uterus (see Hunter, 1974).

The isthmus of the oviduct is believed to function as a sperm reservoir in some primates, rabbits, rodents, pigs and ruminants (see Overstreet et al., 1989). Movement of spermatozoa from the isthmus to the ampulla is dependent on ovulation and is probably due to a combination of hyperactivated sperm motility and ovulation-related changes in the contractility of the oviducal musculature (see Overstreet & Katz, 1990).

1. 3. 2. 2. Domestic Birds

During copulation, between 500 and 1500 X 10⁶ spermatozoa are believed to be deposited in the everted posterior vagina (Lake, 1975). Chicken spermatozoa are vigorously motile upon ejaculation and require to be motile in order to migrate through the vagina towards the uterovaginal junction (Fujii & Tamura, 1963; Allen & Grigg, 1957; Schindler et al., 1967, Takeda, 1974).

The vast majority of spermatozoa reaching the uterovaginal junction, following either intravaginal insemination or natural mating, enter the uterovaginal junction SSTs and are subsequently stored there. Only very small numbers of spermatozoa migrate directly to the anterior regions of the oviduct, and only occasionally are spermatozoa observed in the infundibular SSTs following vaginal deposition (Bohr et al., 1964a). It has been shown that chicken spermatozoa can traverse the vagina and enter the uterovaginal junction SSTs in as little as 15 minutes (Allen & Grigg, 1957; Bohr et al., 1964b). Furthermore, small numbers of spermatozoa have been observed in the anterior regions of the oviduct one hour following intravaginal insemination (Allen & Grigg, 1957; Saeki et al., 1963).

Beyond the uterovaginal junction, sperm transport is believed to be passive (Fujii & Tamura, 1963; Van Krey et al., 1967). Evidence to support this postulation was

provided by Allen and Grigg (1957) and Saeki et al. (1963), who observed that dead spermatozoa introduced into the uterus subsequently appeared in more anterior regions of the oviduct. Furthermore, Bakst (1983) observed that turkey spermatozoa inseminated surgically into the magnum and infundibulum did not generally move in an abovarian direction. Passive transport of spermatozoa to the anterior regions of the oviduct is believed to be due to muscle contractions and ciliary activity (Burke et al., 1969).

1. 3. 3. Oviducal Sperm Selection

1. 3. 3. 1. Mammals

Only a very small fraction of spermatozoa in the mammalian ejaculate (<0.001%) reach the site of fertilisation (see Thibault, 1973; Austin, 1975). Some spermatozoa are rapidly evacuated to the exterior by mucus flow and urine flush (Austin, 1975; Morton & Glover, 1974; Overstreet, 1983), the majority of the remainder being eliminated during migration through the oviduct.

Many hypotheses have been advanced to explain the massive wastage of mammalian ejaculated spermatozoa.

Polyspermy is the entry of several spermatozoa to the ovum, resulting in polyploid zygotes, which are either arrested at an early stage of development, or undergo abnormal embryonic development. In mammals, changes in the egg plasma membrane surface and the permeability of the zona pellucida, following penetration by a single spermatozoon, are designed to prevent polyspermy (see Longo, 1987; Austin, 1975). Despite this, there is also a requirement to control the numbers of spermatozoa reaching the site of fertilisation (see Longo, 1987).

Symons (1967) suggested that spermatozoa rejected by the oviduct are senescent. Johnson (1973) and Hancock (1984) have suggested that excess spermatozoa are removed,

thereby preventing immunological sensitisation of the female to the antigenically foreign spermatozoa. Cohen (1967, 1969, 1973, 1975) has postulated that oviducal sperm selection may have a genetic basis, and that only a very small subpopulation of genetically 'fit' spermatozoa are allowed to proceed to the site of fertilisation.

Baker and Bellis (1988) in their 'Kamikaze Sperm' hypothesis, have postulated that spermatozoa in animal ejaculates are polymorphic and adapted to a variety of roles. Some spermatozoa are egg-fertilisers, the remainder occupying strategic positions in the oviduct where they attempt to prevent the passage of spermatozoa inseminated by other males. Although some Kamikaze spermatozoa may be easily recognisable e.g. those with abnormal morphology, some may be morphologically normal, and egg-fertilisers may differ from others only in their physiology and behaviour.

A considerable amount of work has been carried out implicating the biophysical interaction between mammalian spermatozoa and the environment of the female reproductive tract in sperm selection (see Mortimer, 1978; Overstreet, 1983; Katz et al., 1989; Overstreet & Katz, 1990), with oviducal sperm transport being dependent on the physical properties of cervical and oviducal mucus, the properties of the sperm surface, and the effects of epithelial surfaces on sperm motion.

The viscosity of cervical mucus and other polymeric fluid secretions of the oviduct can influence sperm flagellar motion and the properties of the sperm surface are known to influence penetration of cervical mucus (see Katz et al., 1989). Morphological abnormality of the sperm surface has been reported to restrict cervical mucus penetration (Hanson & Overstreet, 1981; Freundl et al., 1988), probably due to a combination of inferior motility and surface interactions with mucus macromolecules (see Katz et al., 1989). In the cervix and lower oviduct, sperm adherence to epithelial surfaces is believed to play a role in the regulation of sperm transport (Overstreet, 1983; Suarez, 1987). This may also be the case in the lower isthmus (Cooper et al., 1979), caudal isthmus (Smith et al., 1987), and uterotubal junction (Suarez, 1987).

It has been established that cumulus cells play a selective role against abnormal spermatozoa (Katz et al., 1989), and a similar sperm selecting role has been suggested for the zona pellucida (see Menkveld et al., 1991).

A large body of evidence exists for the presence of an immunologically-based oviducal sperm selection mechanism in mammals. Cinader and de Weck (1976) found antibody activity against bacteria, viruses, protozoa, ABO blood group antigens and spermatozoa in the uterine, cervical and vaginal secretions of women, and it has been known for some time that the cervical mucus of many mammals contains

high concentrations of IgA and IgG (Austin, 1960; Bedford, 1965; Parish et al., 1967) which, when bound to the sperm surface can restrict cervical mucus penetration (see Katz et al., 1989). Parish et al. (1967) demonstrated a higher antibody titre in cervical mucus than in blood serum, suggesting a localised immune response within the oviduct. This was confirmed by Waldman et al. (1972) and Menge and Behrman (1980), who observed a local immune response within the oviduct in the absence of a systemic response.

The majority of inseminated spermatozoa are coated by antibody in the cervix (Austin, 1960; Symons, 1967; Cohen & Werrett, 1975; Cohen & Tyler, 1980). It is well established that the fate of the majority of spermatozoa not mechanically expelled soon after copulation or by artificial insemination is phagocytosis in the vagina (Austin, 1975), cervix (Mattner, 1969), and uterus (Austin, 1960, 1975; Yanagimachi & Chang, 1963). Tyler (1977) observed sperm-induced cervical leukocytosis in the rabbit 30 minutes after mating. Sperm induced leukocytosis has also been observed in the rabbit vagina (Phillips & Mahler, 1977), human cervix (Pandya & Cohen, 1985), and canine vagina (Cohen, 1988) within a few minutes of sperm deposition. Taylor (1982b) carried out a double mating experiment with rabbits and observed that spermatozoa introduced to the previously primed and leukocyte dominated female reproductive tract could still fertilise, suggesting a selective phagocytosis.

Smallcombe and Tyler (1980) demonstrated that sperm induced leukocytosis is accompanied by a considerable transfer of immunoglobulins from serum into the vaginal, cervical and uterine fluids. However, Cohen and McNaughton (1974), Cohen and Werrett (1975), Cohen and Gregson (1978), and Cohen and Tyler (1980) reported that the first few spermatozoa through the cervix are not coated with IgG, in contrast to the majority of spermatozoa in the ejaculate. Such spermatozoa are considerably more fertile than spermatozoa from the ejaculate, only 100-150 non-IgG-coated spermatozoa recovered from the oviduct being required to fertilise an egg, compared with 10^4 - 10^5 ejaculated spermatozoa. Sinosich and Saunders (1987) found pregnancy-associated plasma protein A (PAPP-A), which inhibits leukocyte elastase, on the surface of 1.7% of ejaculated spermatozoa, and have suggested that such spermatozoa are selected to overcome localised phagocytic proteolytic degradation.

1.3.3.2. Domestic Birds

In domestic birds, more than 80% of artificially inseminated spermatozoa are mechanically ejected from the cloaca within 15 minutes of insemination (Howarth, 1971) with approximately 1% of inseminated spermatozoa subsequently found in the uterovaginal junction SSTs (Brillard & Bakst, 1990).

Ova of domestic birds are large and can accommodate some supernumery spermatozoa without polyploidy occurring. Bekhtina (1966) reported the presence of up to 200 spermatozoa in fertilised chicken eggs. Numbers exceeding 200 per egg were related to a higher than normal incidence of abnormal embryonic development. It has been demonstrated that the introduction of abnormally high numbers of chicken spermatozoa to the anterior oviduct results in a high frequency of early embryonic death over a sustained period (Ogasawara & Lorenz, 1966; Lorenz & Ogasawara, 1968). Furthermore, Tanaka and Koga (1971) artificially inseminated chicken hens with the uterovaginal junction removed and found a high incidence of embryonic mortality. However, it remains to be elucidated whether increased embryonic mortality observed in such experiments is due to the number or quality of spermatozoa at the site of fertilisation.

Little work has been carried out to identify the physiological basis of oviducal sperm selection in domestic birds. Traditionally the uterovaginal junction SSTs have been regarded as selective with respect to the spermatozoa which enter them, since subfertile spermatozoa have poor access to these tubules in-vivo (Allen & Bobr, 1955; Allen & Grigg, 1957; Ogasawara et al., 1966; Bakst & Bird, 1987). Sperm morphology has been suggested as a basis for sperm selection at the uterovaginal junction SSTs (Ogasawara et al., 1966), as has the idea of a

recognition system between the SSTs and the sperm surface (Lake & Ravie, 1988).

Bakst (1989) and Takeda (1974) have suggested that an element of sperm selection might take place within the vagina. Takeda (1974) suggested that sperm selection in the chicken oviduct is based on motility. However, although under some experimental conditions the motility of chicken spermatozoa measured in-vitro can be highly correlated with their fertilising ability in-vivo (Wishart & Palmer, 1985), evidence also exists to suggest that motility can remain relatively unchanged while fertilising ability is diminished or totally absent. Conditions resulting in such observations include γ -irradiation of spermatozoa (Wishart & Dick, 1985), treatment of spermatozoa with neuraminidase (Froman & Thurston, 1984; Lake & Ravie, 1988; Froman & Engel, 1989; Howarth, 1989), aerobic incubation leading to lipid peroxide formation by spermatozoa (Wishart, 1984b), and intravaginal insemination of testicular spermatozoa (Munro, 1938b; Howarth, 1983). In some of these cases loss of fertilising ability may be a surface phenomenon: the site of neuraminidase activity is probably terminal sialic acid residues on sperm surface glycoproteins (Froman & Engel, 1989), and testicular spermatozoa present a surface which is antigenically dissimilar to that of ejaculated spermatozoa (Esponda & Bedford, 1985).

1.3.4. Oviducal Sperm Storage

1.3.4.1. Mammals

The periods during which spermatozoa maintain their fertility and viability in the mammalian oviduct are extremely variable between species. Spermatozoa from some species lose their fertilising ability quickly e.g. mouse (6 hours), rat (14 hours) and hamster (21 hours), but most, including bovine, porcine, ovine, rabbit and ferret spermatozoa remain fertile for up to 48 hours in the oviduct (Blandau, 1969). This period is extended to around 80 hours for human (see Zinaman et al., 1989), 120 hours for llama (see Thibault, 1973), 144 hours for equine (Blandau, 1969) and 168 hours for canine (see Thibault, 1973) spermatozoa. Loss of fertilising ability generally precedes loss of sperm viability. For example, motile human spermatozoa have been recovered from the cervix 8 days after coitus (see Zinaman et al., 1989) and bovine spermatozoa have been similarly recovered 4 days following insemination (Blandau, 1969). Prolonged sperm storage is observed in the hare (>41 days; see Thibault, 1973) and in a few species of bat which mate in autumn and ovulate in spring (Racey, 1973).

Mammalian spermatozoa are stored at various oviducal sites, including the isthmus e.g. llama; uterotubal junction e.g. hare, cow; uterine lumen e.g. pipistrelle bat, dog; and uterine glands e.g. dog, bat, guinea pig,

mouse, stoat, ewe and cow (see Thibault, 1973). Furthermore, the mucus-filled cervix is considered to be a sperm reservoir in domestic farm animals and primates (see Overstreet et al., 1989), including women (Zinaman et al., 1989). However, anatomical differentiation of storage sites has only been described in the camelidae e.g. llama, in which the enlarged tubal isthmus acts as a sperm reservoir (see Thibault, 1973).

1. 3. 4. 2. Domestic Birds

Chicken spermatozoa are believed to gain entry to uterovaginal junction SSTs by a combination of their own motility and ciliary activity at the orifice of the SSTs (J. P. Brillard, personal communication). Sperm storage at the uterovaginal junction SSTs in chickens provides an extended fertile period of around 19 days (see Thibault, 1973).

Bakst and Richards (1985) postulated that prolonged survival of spermatozoa in the uterovaginal junction SSTs may involve the reversible suppression of sperm metabolism and motility, stabilisation of plasma and acrosomal membranes, inhibition of acrosomal enzymes, and suppression of an immune response to the cells. The SST cells are believed to supply oxidisable substrates to the spermatozoa and to remove the waste products of sperm

metabolism (Van Krey et al., 1967; Fujii, 1963; Friess et al., 1978), probably via diffusion of secretions.

Spermatozoa stored in the uterovaginal junction SSTs are immotile (Bakst, 1987a) and are orientated towards the base of the SST, exhibiting head to head agglutination (Van Krey et al., 1981). These authors, and more recently Froman and Thurston (1984), Froman and Engel (1989), and Howarth (1989) have suggested that agglutination may be the basic mechanism controlling sperm storage.

There have been conflicting reports regarding the timing of release of spermatozoa from the uterovaginal junction SSTs. Van Krey (1964) and Bobr et al. (1964b) suggested that release of spermatozoa is restricted to the period immediately following oviposition. However, Burke and Ogasawara (1969) reported the presence of spermatozoa in the vaginal fluids of hens before and after oviposition, and Bakst (1981) reported the presence of spermatozoa in the lumen of the turkey oviduct irrespective of the stage of the ovulatory cycle, suggesting a continuous release.

Grigg (1957) suggested that sperm release from the infundibular SSTs is due to stretching of the oviduct by passage of the ovum. However, Van Krey (1964) demonstrated that this is not the case regarding sperm release from the uterovaginal junction SSTs. Van Krey et al. (1967) reported a lack of myoepithelial cells associated with the SSTs, ruling out tubular contraction

as a sperm releasing mechanism. Zavaleta and Ogasawara (1987) have suggested that spermatozoa are gently flushed out of SSTs with their flagellae foremost (Van Krey, 1964) during periodic peaks of SST secretory activity.

1. 3. 5. Capacitation

1. 3. 5. 1. Mammals

In addition to spermatogenesis and epididymal maturation, mammalian spermatozoa require an additional phase of maturation within the female reproductive tract before they can acrosome react and fertilise ova (see Austin, 1951; Yanagimachi, 1981; Moore & Bedford, 1983). This maturation process, termed capacitation, entails a series of biochemical and biophysical changes, including the development of hyperactivated motility (see Yanagimachi, 1981), increase in plasma membrane permeability to Ca^{2+} (Singh et al., 1978), activation of adenylate cyclase and acrosomal enzymes (see Clegg, 1983), modification of the sperm plasma membrane including rearrangement of plasma membrane antigens and other components (Koehler, 1978), loss of surface associated inhibitory components (Fraser, 1984), changes in plasma membrane phospholipid composition (Davis et al., 1980), a decrease in net negative surface charge (see Longo, 1987), and changes in lectin binding to the sperm surface (Koehler, 1978; Cross & Overstreet, 1987). It has been suggested that elevated Ca^{2+} levels may regulate modification of the sperm plasma membrane and acrosomal membrane, the activities of acrosin and adenylate cyclase, and sperm motility, via calmodulin (see Longo, 1987).

The site of sperm capacitation in mammals varies among species. In species where semen is deposited in the uterus, capacitation occurs primarily in the oviduct (Moore & Bedford, 1983). Where semen is deposited in the vagina, capacitation starts there and continues as spermatozoa migrate to the anterior regions of the oviduct (Zinaman et al., 1989).

1. 3. 5. 2. Domestic Birds

The situation regarding capacitation in domestic birds is believed to be entirely different from that known to exist in mammals. Olsen and Neher (1948), and later Bobr et al. (1964b) and Nishiyama et al. (1968) observed that spermatozoa inseminated directly into the infundibulum just prior to ovulation fertilised eggs. Howarth (1971) obtained fertilised newly ovulated chicken ova from a 15 minute incubation with spermatozoa in-vitro. This was repeated by Fujihara et al. (1973), and by Howarth and Palmer (1972) for turkey, suggesting that capacitation is not a requirement for spermatozoa from domestic birds. In support of this hypothesis, Dukelow et al. (1967) failed to find a decapacitation factor in rooster semen.

The possibility of parthenogenesis resulting from in-vitro fertilisation seems unlikely, given that Olsen (1966) reported that only 4 out of 5931 eggs exhibited parthenogenetic development.

1. 3. 6. Sperm-Egg Interaction

1. 3. 6. 1. Mammals

The mammalian zona pellucida comprises three glycoproteins, ZP1, ZP2 and ZP3, in an interconnected three dimensional lattice (see Wassarman, 1992).

In-vitro studies have demonstrated the presence of receptors on the sperm plasma membrane for zona components (Storey et al., 1984), located over the equatorial and postacrosomal regions (O'Rand & Fisher, 1987). Spermatozoa undergo the acrosome reaction, a form of cellular exocytosis required for spermatozoa to penetrate the zona pellucida and fuse with the egg plasma membrane after binding to the zona pellucida (Storey et al., 1984; Wassarman, 1992). ZP3 is responsible for sperm-binding and induction of the acrosome reaction (Bleil & Wassarman, 1980, 1983; Florman & Wassarman, 1985). However, there is evidence to suggest that spermatozoa from some species may undergo the acrosome reaction prior to zona pellucida binding (Huang et al., 1981).

Sperm receptor activity of ZP3 is dependent upon its carbohydrate content, whereas the induction of the acrosome reaction is dependent upon the polypeptide chain also (Florman et al., 1984; Florman & Wassarman, 1985).

Mouse spermatozoa possess galactosyltransferase which is activated during capacitation (see Shur, 1989). Galactose residues are lost from the active site of the enzyme, allowing zona pellucida binding, probably to O-linked oligosaccharides covalently bound to ZP3 (Florman et al., 1984; Florman & Wassarman, 1985). Acrosome reacted spermatozoa bind to ZP2 in the mouse (see Wassarman, 1992).

Entry of the spermatozoon to the egg is followed by a rapid depolarisation of the egg plasma membrane, which reduces the receptivity of the egg to further sperm fusion, and constitutes the rapid, partial block to polyspermy (see Longo, 1987). This is followed by the discharge of vesicles from the egg cortex - the cortical granule reaction. Peroxidase activity associated with the cortical granules alters the zona pellucida, making it more resistant to solubilisation (Schmell & Gulyas, 1980). In the mouse, ZP3 and ZP2 are inactivated as primary and secondary receptors. ZP3 is modified by a cortical granule glycosidase and ZP2 is modified by a cortical granule protease (see Wassarman, 1992).

The proteins on the sperm surface responsible for zona pellucida and egg plasma membrane binding have not yet been identified. However, in the mouse, evidence exists for the location of the ZP3 binding protein in the plasma membrane overlying the sperm head, and the ZP2

binding protein in the inner and outer acrosomal membrane (see Wassarman, 1992).

1. 3. 6. 2. Domestic Birds

Little is known about sperm-egg interaction in domestic birds. Bellairs et al. (1963) and Fujii (1976) suggested that chicken spermatozoa might penetrate the inner perivitelline layer by passing between the fibres comprising this investment. However, Bakst & Howarth (1977b) identified a ground substance occupying the apparent spaces between fibres.

Chicken spermatozoa possess acrosomal trypsin-like enzymic activity (Langford & Howarth, 1974; Brown & Hartree, 1976), which hydrolyses the inner perivitelline layer of the egg (Ho & Meizel, 1975; Bakst & Howarth, 1977b; Okamura & Nishiyama, 1978). The inner perivitelline layer of the egg is believed to promote the acrosome reaction (Koyanagi et al., 1988), with the subsequent digestion of a pathway to the egg plasma membrane (Bakst & Howarth, 1977b; Okamura & Nishiyama, 1978). The inner perivitelline layer overlying the animal pole is reported to be preferentially hydrolysed in-vitro (Howarth & Digby, 1973; Ho & Meizel, 1975; Bakst & Howarth, 1977b), although there is no apparent difference in the composition of the animal and vegetal poles (Bakst & Howarth, 1977b).

Based on results obtained from the in-vitro incubation of chicken spermatozoa with inner perivitelline layer isolated from chicken eggs, Howarth (1990) has suggested that in the chicken, sperm-egg interaction is a specific, receptor-mediated process. Furthermore, Howarth (1992) has recently reported the involvement of N- and O- linked oligosaccharides on inner perivitelline layer components, in sperm-egg interaction.

Bakst and Howarth (1977b) have postulated that the outer perivitelline layer may have a role in blocking pathological polyspermy.

1. 4. Interspecies Fertilisation

1. 4. 1. Mammals

Species specificities are believed to exist at all levels in the reproductive process, the strictest being geographical isolation and mating behaviour of different species (O'Rand, 1986), and fertilisation does not normally occur between intact gametes of phylogenetically distant species (Roldan & Yanagimachi, 1989). However, strict specificity does not appear to exist at the level of gamete interaction (O'Rand, 1988), and it is believed that the more closely related are the species, the greater are the chances of cross-fertilisation occurring (Maddock & Dawson, 1974; Roldan et al., 1985).

Heterologous combinations between mouse, rat, Syrian hamster or rabbit gametes do not result in fertilisation in-vitro (Dickmann, 1962; Yanagimachi, 1964, 1972; Barros, 1968; Hanada & Chang, 1976), although heterologous fertilisation can occur from the interaction of gametes from closely related species in-vitro or in-vivo (Maddock & Dawson, 1974; Roldan et al., 1985).

In species with internal fertilisation, heterologous gamete combinations are always less successful than homologous gamete combinations (Roldan et al., 1985) and the female reproductive tract is believed to play an important role as a barrier to interspecies fertilisation

(see O'Rand, 1988), possibly due to the effect of interactions of spermatozoa with the female environment on sperm viability, the timing of capacitation, and the timing of the acrosome reaction (Cummins & Yanagimachi, 1986; Smith et al., 1988).

Barriers to interspecies fertilisation exist at both the zona pellucida and the plasma membrane (Yanagimachi, 1977; Hanada & Chang, 1978), although in many mammalian species e.g. Syrian hamster, rat and rabbit, species specificity occurs primarily at the zona pellucida, but less so at the plasma membrane (Yanagimachi, 1972; Yanagimachi et al., 1976; Hanada & Chang, 1976; Roldan & Yanagimachi, 1989). The opposite situation applies to Chinese hamster eggs, in which the plasma membrane constitutes the main barrier to interspecies fertilisation (Roldan & Yanagimachi, 1989). In other mammals, e.g. closely related deermouse species, interspecies fertilisation readily occurs (Fukuda et al., 1979), suggesting limited species specificity at both the zona pellucida and plasma membrane.

1.4.2. Domestic Birds

In domestic birds there has as yet been no investigation of barriers to interspecies fertilisation at the level of sperm-oviduct interaction or sperm-egg interaction, except that of Takeda (1974) who observed that bovine spermatozoa inseminated intravaginally into chicken hens did not

migrate to the uterovaginal junction. However, there is evidence to suggest incomplete species specificity in closely related avian species. Small numbers of hybrids have been obtained from the insemination of quail hens with chicken semen (Ogasawara & Huang, 1963; McFarquhar & Lake, 1964; Mathis & McDougald, 1987), chicken hens with guinea fowl semen (Mathis & McDougald, 1987; Steklenev & Kozikova, 1989), pheasant hens with chicken semen (Watanabe, 1964), and natural mating of peafowl with guinea fowl (Mathis et al., 1983).

1. 5. Aims of the Work

1. To identify proteins and glycoproteins, secreted by the excurrent duct system of the testes, which associate with the chicken sperm surface and to characterise changes occurring to proteins and glycoproteins on the sperm surface and in seminal plasma during transport through the excurrent duct system.
2. To determine the role of sperm surface associated proteins in oviducal sperm transport.
3. To identify the site and physiological basis of oviducal sperm selection.
4. To investigate whether sperm surface changes are induced by the oviduct prior to fertilisation.
5. To investigate the specificity of sperm-egg interaction, and the efficiency of the inner perivitelline layer as a barrier to interspecies fertilisation in closely and distantly related avian species.
6. To elucidate the relative importance of the vagina and inner perivitelline layer of the egg

as barriers to interspecies fertilisation
between closely and distantly related avian
species.

CHAPTER 2. MATERIALS AND METHODS

2. MATERIALS AND METHODS

2.1. Bird Maintenance and Semen Collection

Male chickens were a Rhode Island Red-like control strain from Ross Breeders Ltd., U.K.; chicken hens were 'I.S.A. Brown' commercial layers; male turkeys were the progeny of a cross between British United Turkeys strains 5 and 6; and male and female Japanese quail were from a breeding colony at Dundee Institute of Technology. All birds were housed individually, given 14 hours light, 10 hours dark photoperiod and fed a commercial breeders ration ad-libitum.

Semen was collected from chickens and turkeys as described by Wishart (1982) and from quail by the method of Ogasawara and Huang (1963). For most experiments, pooled ejaculates from between 4 and 8 individuals from a given species were used.

2. 2. Investigation of Sperm Surface and Seminal Plasma Changes Occurring During Post-testicular Maturation in the Chicken

2. 2. 1. Detection of Maturation Related Sperm Surface Changes

2. 2. 1. 1. Production and Testing of Antiserum Raised in the Rabbit against Chicken Ejaculated Seminal Plasma

Semen was collected from birds and pooled as previously described (2.1.), centrifuged (I.E.C. CENTRA-4R) at 700 g for 15 minutes at 5 °C, and then microcentrifuged (MSE MICROCENTAUR) at 13000 g for 10 minutes to remove any remaining spermatozoa.

Rabbits (New Zealand White strain) were injected subcutaneously into the back with 400 µl per site over 5 sites, with an emulsion comprising equal parts of Freund's Complete Adjuvant (Sigma Chemical Co.) and seminal plasma, and subsequently 21 days later with a similar emulsion comprising Freund's Incomplete Adjuvant (Sigma Chemical Co.) and seminal plasma (Harlow & Lane, 1988).

Test bleeds were obtained from the marginal ear vein 12 days following booster injection, providing approximately 15-20 ml of blood. After incubation at

37 °C for one hour, the clot was separated from the wall of the collecting tube and allowed to contract overnight at 4 °C. The serum was removed from the clot, microcentrifuged at 13000 g for 10 minutes, divided into 100 µl aliquots and stored at -20 °C.

The assay used to determine antiserum titre was an adaption of the Macroscopic Sperm Agglutination Test (Kibrick et al., 1952). An aliquot of 100 µl of chicken semen was washed twice in 5 ml of buffer comprising 0.15M NaCl with 20mM N-Tris-[hydroxymethyl]-methyl-2-aminoethanesulphonic acid (TES; Sigma Chemical Co.), pH 7.4 (NaCl/TES), resuspended in 100 µl of 0.15M NaCl/TES and a sperm count performed using a haemocytometer. Spermatozoa were subsequently diluted to 50×10^6 /ml with 0.15M NaCl/TES, and 200 µl added to 200 µl of antiserum diluted 1:10, 1:100, 1:500 and 1:1000 in 4 ml polycarbonate tubes. Control incubations were carried out using normal rabbit serum.

After mixing by inversion, sperm suspensions were incubated at 37 °C for 60 minutes and checked for sedimentation and loss of turbidity.

2. 2. 1. 2. Protocol for Semen Preparation and
Immunofluorescent Staining of Spermatozoa

Individual ejaculates were obtained from three cockerels, which were subsequently sacrificed by cervical dislocation and the entire reproductive tracts carefully excised. The testes were separated from the epididymal region and immersed in 0.15M NaCl/TES. All tissue surrounding the ductus deferens was carefully removed, and the proximal and distal 100 mm portions of the ductus deferens removed separately into 0.15M NaCl/TES. Spermatozoa were obtained from these tissues by squeezing the luminal contents into a single drop of 0.15M NaCl/TES. The testes were halved and laid with their cut edge downwards into a small volume of 0.15M NaCl/TES, gentle pressure applied to the tissue, and fluid containing spermatozoa collected by Pasteur pipette.

Sperm counts were performed and the sperm concentrations adjusted to 5×10^6 spermatozoa in 500 μ l of 0.15M NaCl/TES containing 4% formaldehyde (v/v). After fixation for 20 minutes at room temperature, sperm suspensions were centrifuged at 700 g for 10 minutes at 5 °C, washed with 500 μ l of 0.15M NaCl/TES, and resuspended in 50 μ l of 0.15M NaCl/TES.

Non-specific binding sites were blocked with 200 μ l of normal goat serum diluted 1:10 for 30 minutes at 37 °C. The sperm suspensions were centrifuged at 700 g for

10 minutes at 5 °C, washed with 500 µl of 0.15M NaCl/TES and the pellet resuspended in either the antiserum raised against seminal plasma proteins or control rabbit serum, diluted 1:10 with 0.15M NaCl/TES. After incubation for 30 minutes at 37 °C, spermatozoa were washed twice with 500 µl of 0.15M NaCl/TES and incubated with fluorescein isothiocyanate (FITC)-conjugated goat antirabbit IgG (Sigma Chemical Co.) diluted 1:50 with 0.15M NaCl/TES for 30 minutes at 37 °C. Spermatozoa were subsequently washed twice with 500 µl of 0.15M NaCl/TES, resuspended in 50 µl of 0.15M NaCl/TES and examined for changes in staining pattern and intensity related to post-testicular maturation, using a Leitz Laborlux K microscope equipped with epifluorescence optics and appropriate filters for FITC, at X400 and X1000 (oil) magnification.

2.2.1.3. Protocol for Lectin Staining of Spermatozoa

FITC-conjugated lectins used were obtained from Sigma Chemical Co.. Stock solutions were 1 mg/ml in 0.15M NaCl/TES, pH 7.4 for lectin from Triticum vulgaris (WGA), lectin from Solanum tuberosum, lectin from Erythrina corallodendron, and Peanut Agglutinin, and 0.15M NaCl/TES, pH 7.4 containing 0.1mM CaCl₂ and 0.1mM MnCl₂ for ConA (see Appendix 1 for lectin specificities).

Semen was collected as described in 2.2.1.2. and sperm concentrations adjusted to 2×10^6 in 500 µl of 0.15M

and resuspended in 90 μ l of 0.15M NaCl/TES containing 0.1mM CaCl_2 and 0.1mM MnCl_2 held at 5 $^{\circ}\text{C}$.

The protocol for cell staining was essentially that described by Cross and Overstreet (1987) for unfixed human spermatozoa. In this work, fixed and unfixed spermatozoa were stained using the same protocol.

An aliquot of 5 μ l of stock lectin solution held at 5 $^{\circ}\text{C}$ was added to 45 μ l of sperm suspension in a 4 ml polycarbonate tube, thoroughly mixed by gentle agitation, and incubated at 5 $^{\circ}\text{C}$ for 30 minutes. Thereafter, 450 μ l of 0.15M NaCl/TES containing 0.1mM CaCl_2 and 0.1mM MnCl_2 was added, and the spermatozoa pelleted by centrifugation at 700 g for 10 minutes at 5 $^{\circ}\text{C}$, washed with 500 μ l of 0.15M NaCl/TES containing Ca^{2+} and Mn^{2+} , and finally resuspended in 50 μ l of the same buffer.

Spermatozoa were subsequently examined by epifluorescence microscopy at X400 and X1000 (oil) magnification for changes in staining pattern and intensity related to post-testicular maturation.

2.2.2. Detection of Maturation Related Changes in Seminal Plasma and Sperm Surface-Associated Proteins

2.2.2.1. Seminal Plasma Preparation and Hypertonic Buffer Treatment of Spermatozoa

Ejaculated semen and semen from the anterior and posterior ductus deferens were collected from 5 cockerels as described in 2.2.1.2. and pooled. The sperm concentrations were adjusted to 400×10^6 in 200 μ l of 0.15M NaCl/TES, and the sperm suspensions centrifuged at 700 g for 10 minutes at 5 °C, then microcentrifuged at 13000 g for 10 minutes and stored at -20 °C prior to electrophoresis.

The spermatozoa were resuspended in 1.0 ml of 0.15M NaCl/TES, centrifuged at 700 g for 10 minutes at 5 °C, washed with 1.0 ml of 0.15M NaCl/TES and resuspended in 200 μ l of 1.0M NaCl/TES in 4 ml capped polycarbonate tubes. After gentle agitation for 15 minutes at room temperature the sperm suspensions were centrifuged at 700 g for 10 minutes at 5 °C and the supernatants microcentrifuged at 13000 g for 10 minutes prior to storage at -20 °C.

2.2.2.2. SDS-PAGE of Seminal Plasma and Sperm Surface Washings, and Protocol for Silver Staining of Separated Proteins

Molecular weight markers (Sigma SDS-VII, SDS-VIIB), ejaculated seminal plasma, anterior and posterior ductus deferens seminal plasmas, and sperm surface washings obtained from treatment of spermatozoa with hypertonic (1.0M NaCl) buffer (2.2.2.1.) were diluted 1:6.7, 1:6.7, 1:1.7, 1:1.7 and 30:1 respectively with 0.15M NaCl/TES, and then to 1:20, 1:20, 1:5, 1:5 and 10:1 with boiling mix (see Appendix 2a for composition of electrophoretic reagents). Samples were boiled for 3 minutes, microcentrifuged at 13000 g for 10 minutes and 20 μ l applied to each sample well.

Protein separation was carried out by Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) (Laemmli, 1970) on 5-15% gradient polyacrylamide gels at a constant current of 35mA per gel, using an LKB 2001 vertical electrophoresis unit and LKB 2103 power supply. After separation gels were immediately fixed in a mixture of water : methanol : acetic acid (4:5:1) and subsequently silver stained using the method described by Blum et al. (1987). Gels were washed with 3 changes of 250 ml of a 50% solution of ethanol (v/v) for 60 minutes, and subsequently incubated for one minute in 250 ml of a 0.02% sodium thiosulphate solution (w/v). After rinsing thoroughly with distilled water, gels were incubated with

a staining solution containing 0.02% silver nitrate (w/v) and 0.07% formaldehyde (v/v) for 20 minutes, rinsed thoroughly with distilled water, and the stain developed with 250 ml of a solution containing 6% sodium carbonate (w/v), 0.05% formaldehyde (v/v) and 0.0004% sodium thiosulphate (w/v). After further rinsing, stain development was halted with 250 ml of a solution containing 50% methanol (v/v) and 12% acetic acid (v/v).

Electrophoretic profiles were examined for changes in banding pattern and staining intensity related to post-testicular maturation.

2.2.2.3. Western Blotting and Protocol for Immunological Staining of Blotted Proteins

SDS-PAGE was carried out as described in 2.2.2.2.. Proteins were immediately electroblotted onto Immobilon-P transfer membranes (Millipore) at a constant current of 0.8mA per cm² of gel for 30 minutes, then 1.2mA per cm² for a further 30 minutes, using a Sartorius Sartoblot S-11 blotting apparatus (see Appendix 2b for Western blotting reagents).

The Immobilon membrane was rinsed with phosphate buffered saline (PBS) containing 0.05% Tween 20 (v/v) and non-specific binding sites blocked with PBS containing 0.5% Tween 20 and 10% newborn calf serum (v/v) at 4 °C for

60 minutes, with gentle agitation. After rinsing with PBS/0.05% Tween 20, the membrane was incubated overnight at 4 °C with rabbit antiserum raised against chicken seminal plasma proteins (2.2.1.1.) diluted 1:100 with PBS/0.05% Tween 20 containing 5% newborn calf serum, rinsed with PBS/0.05% Tween 20, and subsequently incubated with horseradish peroxidase (HRP)-conjugated donkey antirabbit IgG (Scottish Antibody Production Unit) diluted 1:200 with PBS/0.05% Tween 20 containing 5% newborn calf serum, for 60 minutes at 4 °C with gentle agitation. After thorough washing with PBS/0.05% Tween 20, the stain was developed with 3,3-diaminobenzidine (DAB) and nickel chloride reagent (0.0006% diaminobenzidine, 0.003% nickel chloride (w/v)) at room temperature (Hsu & Soban, 1982). Staining was halted by washing with PBS.

Blots were subsequently examined for changes in protein antigenicity related to post-testicular maturation.

2.2.2.4. Protocol for Lectin Staining of Blotted Proteins

Electrophoresis and Western blotting were carried out as described in 2.2.2.2. and 2.2.2.3. respectively. Immediately after electroblotting, the membrane was stained with WGA using a protocol based on that described by Kijimoto-Ochiai et al. (1985). After washing with PBS and subsequently PBS/0.5% Tween 20, non-specific binding

sites were blocked with PBS/0.05% Tween 20 for 60 minutes at room temperature with gentle agitation. The membrane was incubated with HRP-conjugated WGA (Sigma Chemical Co.) at a concentration of 20 ug/ml in PBS/0.05% Tween 20 for 60 minutes at room temperature with gentle agitation, thoroughly washed with PBS/0.05% Tween 20 and then with 50mM Tris buffer, pH 7.6. The stain was developed with diaminobenzidine and nickel chloride reagent (2.2.2.3.).

Blots were subsequently examined for changes in the expression of WGA-binding carbohydrate residues related to post-testicular maturation.

2.2.2.5. Protein Band Molecular Weight Determination

Protein band molecular weights were obtained from molecular weight marker (SDSVII, SDSVIIB; Sigma Chemical Co.) calibration curves, and are mean values from 2 gels. Identical proteins on subsequent gels were assigned the mean molecular weight values from the original 2 gels.

2. 3. Investigation of the Influence of Sperm Surface
Modification on Sperm Function

2. 3. 1. Investigation of the Effect of the Removal of
Sperm Surface Associated Proteins by Hypertonic
Buffer Treatment, on Sperm Function In-Vivo and
In-Vitro

2. 3. 1. 1. Hypertonic Buffer Treatment of Spermatozoa, and
Preparation for Assay, Electrophoresis and
Insemination

Ejaculated semen was collected, pooled and 600 μ l diluted with 5 ml of 0.15M NaCl/TES. After centrifugation at 1500 g for 5 minutes at 5 °C and further washing in 5 ml of 0.15M NaCl/TES, the pellet was resuspended in 2 ml of 0.15M NaCl/TES and six 300 μ l aliquots made 0.15, 0.20, 0.25, 0.30, 0.35 and 0.40M with respect to NaCl, by adding 900 μ l of NaCl/TES containing 0.15, 0.22, 0.28, 0.35, 0.42 and 0.48M NaCl respectively. After horizontal incubation with gentle agitation at room temperature (22 °C) for 15 minutes in capped 10 ml polycarbonate vials, the sperm suspensions were 'normalised' to 0.15M NaCl by the slow addition of 2 ml of 0.15, 0.12, 0.09, 0.06, 0.03M NaCl/TES and 20mM TES respectively.

For assays, sperm suspensions were centrifuged at 1500 g for 5 minutes at 5 °C, the pellets resuspended in 800 μ l of 0.15M NaCl/TES, and subsequently incubated in a shaking

waterbath (GALLENKAMP & Co. Ltd) at 150 cycles per minute, for 80 minutes at 40 °C. For insemination, spermatozoa were resuspended in 200 µl of 0.15M NaCl/TES, held at room temperature and inseminated within 20 minutes of preparation. To prepare samples for electrophoresis, an identical treatment protocol was employed but using a 5 fold higher sperm concentration. After hypertonic buffer treatment and normalisation, the supernatants obtained from subsequent centrifugation were microcentrifuged at 13000 g for 10 minutes and stored at -20 °C.

2.3.1.2. Assessment of Sperm Function In-Vitro following Hypertonic Buffer Treatment

2.3.1.2.1. Assessment of Sperm Motility

Collective sperm motility was measured after a 10 minute aerobic incubation at 40 °C by light scattering (Wishart & Ross, 1985). The parameter ODM, which represents the maximum optical density at 550 nm obtained as a suspension of spermatozoa is drawn through a flow cell, was used to estimate concentrations of spermatozoa (Wishart & Palmer, 1985). Sperm motility was defined by the parameter %[ΔODm], which represents the maximum proportional decrease in optical density occurring after movement through the flow cell is arrested, and is correlated with the percentage of motile spermatozoa (Wishart & Ross,

1985). To a 10 ml polycarbonate vial was added 100 μ l of 100mM CaCl_2 in 0.15M NaCl/TES, 5 ml of 0.15M NaCl/TES containing 0.1% bovine serum albumin (fraction V, Sigma Chemical Co.) (w/v), and 200 μ l of sperm suspension. The vial was incubated for one minute at 40 °C and the sperm suspension was drawn through the flow cell. The change in optical density which occurred after stopping the flow of the sperm suspension through the flow cell was measured using a CECIL CE305 spectrophotometer. An output was obtained on a connected flatbed chart recorder (Servoscribe 1B), from which the parameter % $[\Delta\text{ODm}]$ could be calculated.

2.3.1.2.2. Assessment of Sperm ATP Concentration

Sperm ATP concentrations were measured luminometrically in boiled extracts of spermatozoa (Wishart, 1982) after 20 and 80 minutes aerobic incubation. Aliquots of 100 μ l of sperm suspension were added to boiling tubes containing 300 μ l of distilled water, in a boiling waterbath. After a one minute incubation, the tube contents were microcentrifuged at 13000 g for one minute. A stock solution of firefly lantern extract (Sigma Chemical Co.) was diluted 1:15 with distilled water, and 400 μ l added to 50 μ l of boiled sperm extract in a luminometer (Bio Orbit 1250). An output was obtained on a connected flatbed chart recorder (BBC SE120), from which sperm ATP concentrations could be calculated.

2.3.1.2.3. Assessment of Sperm Live/Dead Status

Sperm live/dead status was assessed after a 30 minute aerobic incubation, by staining with nigrosin and eosin (Lake & Stewart, 1978). To 10 μ l of sperm suspension was added 40 μ l of nigrosin and eosin solution (6% nigrosin (w/v), 1.2% eosin (w/v) in 0.15M NaCl/TES). After thorough mixing, 15 μ l was smeared across a microscope slide, immediately air-dried, and examined microscopically at X1000 (oil) magnification.

2.3.1.2.4. Assessment of Sperm Access to the Uterovaginal Junction SSTs In-Vivo

To assess sperm access to the uterovaginal junction SSTs, hens were inseminated either intravaginally or directly into the uterovaginal junction with 20×10^6 spermatozoa. Uterovaginal junction insemination was achieved by determining by digital palpation, the entrance to the uterus and inseminating immediately distal to this location. The hens were sacrificed by cervical dislocation 3 hours post-insemination, and randomly selected portions of the anterior uterovaginal junction mucosa examined as squash preparations by phase contrast microscopy at X1000 magnification. Tissue from the anterior region of the uterovaginal junction was used, since tubules in the anterior region of the uterovaginal junction are less heavily vesiculated, increasing their

transparency and making assessment of luminal contents considerably easier. All SSTs in mucosal biopsies were assessed for the presence of spermatozoa. Smaller numbers of spermatozoa could be counted accurately, but with larger aggregates precise enumeration was not feasible, and therefore SSTs were assigned to groups according to their estimated sperm content.

2.3.1.2.5. Assessment of Sperm Access to the Newly Ovulated Egg In-Vivo

To assess the ability of spermatozoa to gain access to the newly ovulated egg, chicken hens were inseminated intravaginally with 400×10^6 spermatozoa in 50 μ l. The first fertile egg laid approximately 40 hours later was examined by epifluorescence microscopy at X400 magnification for the presence of spermatozoa trapped in the outer perivitelline layer after staining with a 5 μ l/ml solution (in PBS) of the fluorescing nuclear stain 4,6-diamidino-2-phenylindole (DAPI; Sigma Chemical Co.) (Wishart, 1987).

2.3.1.3. SDS-PAGE of Sperm Surface Washings Obtained from Hypertonic Buffer Treatment of Spermatozoa

Molecular weight markers, seminal plasma and sperm surface washings obtained from treatment of spermatozoa with

buffers containing 0.15, 0.20 and 0.25M NaCl were subjected to electrophoresis and silver stained as described in 2.2.2.2..

Electrophoretic profiles were examined for the presence of proteins in sperm surface washings and for evidence of NaCl concentration-dependent protein removal.

In order to highlight differences in band intensities between washings obtained from spermatozoa treated with 0.15 and 0.25M NaCl, gels were scanned using a LKB 2202 laser densitometer.

2.3.1.4. Assessment of the Effect of Re-incubation of Hypertonic Buffer-Treated Spermatozoa with Fresh Buffered Seminal Plasma, on Sperm Access to the Newly Ovulated Egg In-Vivo

Spermatozoa were treated with 0.25M NaCl/TES as described in 2.3.1.1.. Subsequent to hypertonic buffer treatment and normalisation, spermatozoa were centrifuged at 1500 g for 5 minutes at 5 °C and resuspended in 200 µl of either 0.15M NaCl/TES or freshly obtained seminal plasma diluted 1:1 with 0.15M NaCl/TES. Spermatozoa were incubated horizontally with gentle agitation in 4 ml polycarbonate tubes for 15 minutes at 40 °C, and hens were subsequently inseminated intravaginally with 400×10^6 spermatozoa.

Assessment of the ability of spermatozoa to gain access to the newly ovulated egg was carried out as described in 2.3.1.2.5..

2.3.2. Investigation of the Effect of Centrifugation and Washing on Sperm Function In-Vivo and In-Vitro

2.3.2.1. Semen Treatment Protocols and Preparation for Assay and Insemination

Pooled, ejaculated semen was divided into 3 aliquots of 600 μ l, 2 of which were centrifuged at 1500 g for 5 minutes at 5 °C.

Washed Spermatozoa

The pellet was resuspended in 1.2 ml of 0.15M NaCl/TES, centrifuged at 1500 g for 5 minutes at 5 °C, and resuspended in 1.2 ml of the same buffer. The sperm suspension was then divided into 2 aliquots of 600 μ l. For assays, sperm suspensions were incubated in 10 ml polycarbonate vials in a shaking waterbath at 40 °C for 80 minutes.

Unwashed, Centrifuged Spermatozoa

The pellet was resuspended in the original seminal plasma, diluted 1:1 with 0.15M NaCl/TES and centrifuged as above. After resuspension in the same buffered, diluted seminal plasma, the sperm suspension was divided and thereafter treated identically to the washed sperm suspension.

Unwashed, Uncentrifuged Spermatozoa

The final 600 μ l aliquot of neat semen was incubated at 5 °C for 5 minutes then diluted 1:1 with 0.15M NaCl/TES. The sperm suspension was then incubated at 5 °C for 5 minutes, divided and thereafter treated identically to the washed sperm suspension.

For inseminations, spermatozoa were treated as described above, but 1 ml of neat semen was initially used in each case, giving a final volume of 2 ml.

2.3.2.2. Assessment of Sperm Function In-Vitro and In-Vivo Following Centrifugation and Washing

Assays of sperm ATP concentration, motility, live/dead status and access to the newly ovulated egg were carried out as described in 2.3.1.2.1. - 2.3.1.2.3., 2.3.1.2.5.,

with the exception that 100 μ l of sperm suspension was used for the motility assay.

2.3.3. Investigation of the Effect of Neuraminidase Treatment on Sperm Function In-Vivo and In-Vitro

2.3.3.1. Assessment of the Distribution of N-Acetylneuraminic Acid Residues on the Chicken Sperm Surface

Ejaculated spermatozoa were prepared, stained with FITC-conjugated lectin from Limulus polyphemus (Sigma Chemical Co.), specific for N-acetylneuraminic acid residues, and subsequently examined as described in 2.2.1.3..

2.3.3.2. Neuraminidase Treatment of Spermatozoa and Preparation for Assay and Insemination

The method used was essentially that described by Lake & Ravie (1988). Ejaculated semen was divided into 4 aliquots of 200 μ l, and each made up to 1 ml with 0.15M NaCl/TES in 4 ml polycarbonate tubes (2 with buffer at pH 7.4, 2 with buffer at pH 6.0). After thorough mixing by inversion, centrifugation at 1500 g for 5 minutes at 5 °C and further washing in 1 ml of the same

buffer, pellets were resuspended in: - 1) 800 μ l of 0.15M NaCl/TES, pH 6.0; 2) 800 μ l of 0.15M NaCl/TES, pH 7.4; 3) 672 μ l of 0.15M NaCl/TES, pH 6.0 + 128 μ l of a 250 I.U./ml solution of neuraminidase (Type V; Sigma Chemical Co.) in 0.15M NaCl/TES, pH 6.0; or 4) 672 μ l of 0.15M NaCl/TES, pH 7.4 + 128 μ l of a 250 I.U./ml solution of neuraminidase in 0.15M NaCl/TES, pH 7.4. After horizontal incubation with gentle agitation in 4 ml polycarbonate tubes for 10 minutes at 30 °C, spermatozoa were diluted with 2.4 ml of 0.15M NaCl/TES, pH 7.4, centrifuged at 1500 g for 5 minutes at 5 °C, washed with 5 ml of 0.15M NaCl/TES, pH 7.4, resuspended in 1.2 ml of 0.15M NaCl/TES, pH 7.4, and divided into two 600 μ l aliquots for assays.

For inseminations, spermatozoa were finally resuspended in 2 ml of 0.15M NaCl/TES, pH 7.4.

2.3.3.3. Assessment of Sperm Function In-Vitro and In-Vivo Following Neuraminidase Treatment

Assays of sperm motility, ATP concentration, live/dead status, and access to the uterovaginal junction SSTs were carried out as described in 2.3.1.2.1. - 2.3.1.2.4..

2.3.4. Investigation of the Effect of Glycerol on the
Sperm Surface and on Sperm Function In-Vitro

2.3.4.1. Glycerol Treatment of Spermatozoa and
preparation for assay and Electrophoresis

Ejaculated semen was divided into 5 aliquots of 600 μ l, each of which was washed twice with 5 ml of 0.15M NaCl/TES. Pellets were resuspended in 2.4 ml of 0.15M NaCl/TES or 0.15M NaCl/TES containing 0.5, 2, 5 or 8% glycerol (v/v), and incubated horizontally with gentle agitation in 10 ml polycarbonate vials at room temperature (22 °C) for 15 minutes. The sperm suspensions were divided into 1.2 ml aliquots, one of which was centrifuged at 1500 g for 5 minutes at 5 °C and the supernatant microcentrifuged at 13000 g for 10 minutes and stored at -20 °C prior to electrophoresis. The remaining aliquot was made up to 5 ml by the slow addition of 0.15M NaCl/TES, centrifuged at 1500 g for 5 minutes at 5 °C and resuspended in 1.6 ml of 0.15M NaCl/TES. After further division into 800 μ l aliquots, sperm suspensions were incubated in a shaking waterbath at 40 °C for 80 minutes for assays.

An identical protocol was carried out with the entire glycerol treatment protocol at 5 °C, with subsequent aerobic incubation at 40 °C for assays.

2.3.4.2. Assessment of Sperm Function In-Vitro Following Glycerol Treatment

Assays of ATP concentration, motility and eosin exclusion were carried out as described in 2.3.1.2.1. - 2.3.1.2.3. during a 30 minute aerobic incubation at 40 °C, and again after 60 minutes incubation. Sperm morphology was assessed from nigrosin and eosin smears (Lake & Stewart, 1978) at X1000 (oil) magnification after 30 and 60 minutes aerobic incubation at 40 °C, and expressed as the percentage of living spermatozoa retaining normal morphology.

2.3.4.3. SDS-PAGE of Sperm Surface Washings Obtained from Glycerol Treatment of Spermatozoa

Sperm surface washings, ejaculated seminal plasma and molecular weight markers were prepared, subjected to electrophoresis and silver stained as described in 2.2.2.2..

2. 4. Investigation of the Specificity of Protein Association with the Sperm Surface

2. 4. 1. SDS-PAGE of the Soluble and Particulate Fractions of Chicken Ejaculated Seminal Plasma and Sperm Surface Washings

Seminal plasma was prepared from ejaculated semen as described in 2.2.2.1.. The seminal plasma was then centrifuged at 105000 g for 60 minutes at 5 °C. The remaining pellet (particulate fraction) was made up to original volume with 0.15M NaCl/TES. Sperm surface washings were obtained from hypertonic buffer (0.25M NaCl/TES)-treated spermatozoa (2.3.1.1.).

Molecular weight markers, seminal plasma, soluble and particulate fractions of seminal plasma, and sperm surface washings were prepared to final dilutions of 1:20, 1:20, 1:10, 1:1 and 10:1 in boiling mix. Samples were boiled, centrifuged (2.2.2.2.), and 2 μ l aliquots subjected to electrophoresis on pre-cast 10-15% gradient gels (Pharmacia). Gels were silver-stained using the Pharmacia Phastsystem (see Appendix 3 for Phastsystem reagents and automated gel staining protocol).

Electrophoretic profiles were examined for differences in pattern and relative intensity of protein banding.

2.4.2. Protocol for Investigation of the Relative Affinities of Proteins for the Sperm Surface

Ejaculated semen (600 μ l) was added to 5 ml of 0.15M NaCl/TES, centrifuged at 1500 g for 5 minutes at 5 °C, washed with 5 ml of 0.15M NaCl/TES and resuspended in 100 μ l of 0.15M NaCl/TES. A sperm count was performed and the sperm concentration adjusted to 1.5×10^9 sperm in 400 μ l of 0.15M NaCl/TES. After horizontal incubation with gentle agitation in a 4 ml polycarbonate tube at room temperature for 15 minutes, the sperm suspension was centrifuged at 1500 g for 5 minutes at 5 °C and the supernatant microcentrifuged at 13000 g for 10 minutes prior to storage at -20 °C. The pellet was subsequently resuspended in 400 μ l of 1.0M NaCl/TES and incubated for 15 minutes as described above.

Sperm surface washings were diluted to a final concentration of 5:1 in boiling mix. Electrophoresis and silver staining of molecular weight markers, ejaculated seminal plasma, and sperm surface washings obtained from 0.15M and 1.0M NaCl treatment of spermatozoa were carried out as described in 2.2.2.2..

Electrophoretic profiles were examined for differential extraction of proteins from the sperm surface by isotonic and hypertonic buffers.

2. 5. Production of Monospecific Polyclonal Antisera
from Individual Sperm Surface-Associated
Proteins

2. 5. 1. Hypertonic Buffer Treatment of Spermatozoa and
SDS-PAGE of Sperm Surface Washings

Sperm surface washings were obtained by incubating spermatozoa with 1.0M NaCl/TES, following the protocol outlined in 2.4.2.. After electrophoresis (2.2.2.2.), Western blotting and immunological staining (2.2.2.3.) of ejaculated seminal plasma proteins to identify antigenic proteins, electrophoresis of sperm surface washings was carried out (2.2.2.2.), applying 50 μ l per sample well. The unfixed gel was subsequently washed with several changes of distilled water, stained with 0.3M CuCl_2 (see Harlow & Lane, 1988) and viewed against a dark background. Protein bands at 11, 25, 43 and 46 kd were excised from 15 gels and each suspended in 1 ml of 0.15M NaCl/TES using a pestle and mortar.

2. 5. 2. Production, Testing and Preparation of Antisera

Rats (Wistar) were injected intraperitoneally with 1 ml of an emulsion comprising equal volumes of Freund's Complete Adjuvant and protein suspension, and subsequently 21 days later with a similar emulsion comprising equal volumes of Freund's Incomplete Adjuvant and protein suspension.

Tail bleeds performed 10 days following booster injection yielded approximately 500 μ l of blood from which antisera were prepared and tested (2.2.1.1.). Rats demonstrating serum sperm agglutinating activity were anaesthetised (diethyl ether) and exsanguinated by heart puncture. Subsequent preparation and storage of antisera followed the protocol outlined in section 2.2.1.1..

2.5.3. Testing Antiserum Specificity by Immunological Staining of Blotted Seminal Plasma Proteins

Seminal plasma proteins were separated electrophoretically and blotted as described in 2.2.2.2. and 2.2.2.3. respectively. Blotted proteins were stained using the monospecific antisera at a concentration of 1:100, and subsequently HRP-conjugated rabbit antirat IgG (Sigma Chemical Co.) at a concentration of 1:200, as described in 2.2.2.3..

Blots were subsequently examined for cross-reactivity of antisera with other proteins in the seminal plasma profile.

2. 6. Investigation of Oviducal Sperm Selection

2. 6. 1. In-Vitro Investigation of Sperm Selection by
the Uterovaginal Junction SSTs

2. 6. 1. 1. Preparation of Quail Uterovaginal Junction
Tissue

Adult quail hens (in-lay) were sacrificed by cervical dislocation and the uterus and vagina excised. The area of the uterovaginal junction containing SSTs was identified by microscopic examination of mucosal biopsies at X100 magnification.

Two types of aerobic incubation were used which involved either small randomly selected pieces of uterovaginal junction mucosal tissue (approximately 1 mm³) in suspension, or the whole or part of the whole uterovaginal junction. In the former case, randomly selected mucosal tissue pieces were immersed in 3 ml of Earle's Balanced Salt Solution (Flow Laboratories) buffered with 20mM TES, pH 7.4, in 25 ml Nalgene flasks incubated at 40 °C with gentle agitation. In the latter case, tissue was immersed in the same buffer in 35 mm diameter tissue culture dishes (Corning) at 40 °C with the mucosal surface just exposed. Assessment of tissue viability was by periodic observation of tissue ciliary activity (Tanaka & Koga, 1971).

2.6.1.2. Incubation of Quail Uterovaginal Junction Tissue with Homologous and Heterologous Spermatozoa In-Vitro, and Assessment of Sperm Access to the SSTs

Species utilised as sperm donors included quail, chicken, turkey, rat, mouse, bull and human. Of these, chicken, turkey, bull and human spermatozoa were obtained from ejaculates. Spermatozoa were obtained from the epididymis of the mouse and rat by slicing open and squeezing the contents into a small volume of buffered Earle's Balanced Salt Solution, and from the posterior ductus deferens of quail (2.2.1.2.). In addition, spermatozoa from the chicken testis and posterior ductus deferens were used.

Turkey, chicken and human spermatozoa were incubated with uterovaginal junction mucosal tissue pieces. In each case, 10×10^6 spermatozoa in 200 μ l of Earl's Balanced Salt Solution were added, following preincubation of 6 tissue pieces per flask at 40 °C for 10 minutes.

Spermatozoa layered over the uterovaginal junction mucosal surface were obtained from chicken (testis), quail (posterior ductus deferens), chicken (posterior ductus deferens), mouse (epididymis), rat (epididymis), chicken (ejaculate), bull (ejaculate) and human (ejaculate). Tissue was preincubated at 40 °C for 20 minutes, and 100 μ l of sperm suspension layered over the mucosal surface. The number of spermatozoa used varied with

species, being 10×10^6 for human, bull and chicken ejaculated, 20×10^6 for mouse and rat epididymal and chicken testicular, 50×10^6 for chicken posterior ductus deferens and 120×10^6 for quail posterior ductus deferens spermatozoa.

Assessment of sperm access to the uterovaginal junction SSTs was carried out after 60 minutes for both types of incubation. Due to fundamental differences among the spermatozoa used, such as morphology, motility characteristics and the number of spermatozoa incubated, a direct comparison of the numbers of spermatozoa from different species observed per tubule was not feasible. Assessment was made in terms of the percentage of SSTs observed to contain spermatozoa after microscopic examination of squash preparations of uterovaginal junction tissue, at X1000 (oil) magnification.

2. 6. 1. 3. Assessment of the Cross-reactivity of Antiserum, Raised Against Chicken Ejaculated Seminal Plasma Proteins, with Bovine and Human Spermatozoa

Fresh ejaculated bovine and human spermatozoa were prepared and stained as described in 2.2.1.2., and subsequently examined by epifluorescence microscopy at X400 and X1000 (oil) magnification for cross-reactivity of the antiserum with mammalian sperm surface antigens.

2.6.1.4. Incubation of Neuraminidase and Hypertonic Buffer Treated Chicken Spermatozoa with Chicken Uterovaginal Junction Tissue In-Vitro, and Assessment of Sperm Access to the SSTs

Adult chicken hens (in-lay) were sacrificed by cervical dislocation, and uterovaginal junction tissue prepared for non-shaken aerobic incubation as described for quail (2.6.1.1.). Chicken spermatozoa were treated with neuraminidase (2.3.3.2.) or 0.25M NaCl/TES (2.3.1.1.), and 100×10^6 spermatozoa in 100 μ l of 0.15M NaCl/TES layered over the uterovaginal junction mucosa. Control (untreated) spermatozoa were incubated similarly. After a 2 hour incubation at 40 °C, assessment of sperm accessibility to the SSTs was carried out as described in 2.3.1.2.4.. Sperm motility was periodically assessed by microscopy throughout the incubation. Tissue viability was assessed throughout the incubation by microscopic examination of ciliary activity.

2.6.1.5. Incubation of Morphologically Abnormal Chicken Spermatozoa with Quail Uterovaginal Junction Tissue In-Vitro, and Assessment of Sperm Access to the SSTs

Chicken spermatozoa were treated with 0.4M NaCl/TES (2.3.1.1) to produce a high percentage of abnormal forms,

and reconstituted to a final concentration of 100×10^6 spermatozoa in 50 μ l of 0.15M NaCl/TES.

Quail uterovaginal junction tissue was prepared for non-shaken incubation (2.6.1.1.) and incubated with 100×10^6 spermatozoa for one hour (2.6.1.2.). Tissue was prepared for assessment as described in 2.6.1.2., and assessment made in terms of the percentage of SSTs containing abnormal forms.

2.6.1.6. Intravaginal Insemination of Quail Hens with Human Spermatozoa and Assessment of Sperm Access to the Uterovaginal Junction SSTs and Newly Ovulated Egg In-Vivo

Seven quail hens were inseminated intravaginally with 15 μ l of ejaculated human semen (approximately 1.5×10^6 spermatozoa). Two birds were sacrificed 3 hours after insemination, the uterovaginal junctions excised, and SST contents assessed as described in 2.6.1.2.. Eggs were collected from the remaining 5 hens from the second to the fifth day following insemination and checked for the presence of spermatozoa trapped in the outer perivitelline layer (2.3.1.2.5.).

2.6.2. Investigation of the Site of Oviducal Sperm Selection in the Chicken

2.6.2.1. Incubation of Chicken and Turkey Spermatozoa with Chicken Uterovaginal Junction Tissue In-Vitro, and Assessment of Sperm Access to the SSTs

Adult chicken hens (in-lay) were sacrificed by cervical dislocation, and tissue prepared for shaken aerobic incubation as described for quail (2.6.1.1.), with the exception that 18 tissue pieces were incubated per flask. Incubations were carried out as described in 2.6.1.2., using 100×10^6 spermatozoa in 200 μ l of Earle's Balanced Salt Solution. Three pieces of tissue were removed from each incubation after 5, 10, 20, 40, 70 and 100 minutes, immersed briefly in PBS containing 4% formaldehyde (v/v) to immobilise spermatozoa on the exterior of the tissue, and sperm access to the uterovaginal junction SSTs assessed as described in 2.3.1.2.4.. Motility and tissue viability were assessed throughout the incubation as described in 2.6.1.3..

2.6.2.2. Investigation of Antigenic Similarity Between
Chicken and Turkey Spermatozoa

2.6.2.2.1. Assessment of the Cross-reactivity of Antiserum
Raised Against Chicken Ejaculated Seminal
Plasma Proteins, with Turkey Spermatozoa

Chicken and turkey spermatozoa were prepared and stained as described in 2.2.1.2..

Assessment was carried out as in 2.6.1.4..

2.6.2.2.2. Absorption of Antiserum Raised Against Chicken
Ejaculated Seminal Plasma Proteins, with Fixed
Turkey Spermatozoa, and Subsequent Staining of
Chicken Spermatozoa

Turkey semen (1.2 ml) was diluted in 5 ml of 0.15M NaCl/TES, centrifuged at 700 g for 15 minutes at 5 °C and the pellet resuspended in 1.2 ml of 0.15M NaCl/TES. A sperm count was performed and 2×10^9 spermatozoa were fixed in 1 ml of 0.15M NaCl/TES containing 4% formaldehyde (v/v) for 20 minutes at room temperature. The sperm suspension was centrifuged at 700 g for 10 minutes at 5 °C, washed with 1 ml of 0.15M NaCl/TES and non-specific binding sites blocked with 2 ml of normal goat serum diluted 1:10 with 0.15M NaCl/TES for 30 minutes at 37 °C.

After centrifugation at 700 g for 15 minutes at 5 °C, spermatozoa were washed with 1 ml of 0.15M NaCl/TES and incubated with 2 ml of antiserum diluted 1:10 with 0.15M NaCl/TES for 30 minutes at 37 °C. The sperm suspension was centrifuged at 700 g for 15 minutes at 5 °C and the supernatant (antiserum) microcentrifuged at 13000 g for 10 minutes. After washing with 2 ml of 0.15M NaCl/TES, spermatozoa were incubated with 2 ml of FITC-conjugated goat antirabbit IgG diluted 1:50 in 0.15M NaCl/TES for 30 minutes at 37 °C, centrifuged at 700 g for 15 minutes at 5 °C, washed with 2 ml of 0.15M NaCl/TES and finally resuspended in 1 ml of 0.15M NaCl/TES. Spermatozoa were subsequently examined by epifluorescence microscopy at X400 and X1000 (oil) magnification.

The sperm-exposed antiserum was then incubated with a fresh aliquot of 2×10^9 fixed, blocked turkey spermatozoa and the staining procedure repeated 5 times until sperm fluorescence was no longer detectable. Chicken spermatozoa were subsequently stained with untreated and absorbed antisera according to the protocol described in 2.2.1.2. and examined by epifluorescence microscopy at X400 and X1000 (oil) magnification for differences in staining related to antiserum absorption.

2.6.2.3. Intravaginal and Uterovaginal Junction
Insemination of Chicken Hens with Chicken and
Turkey Spermatozoa, and Assessment of Sperm
Access to the Uterovaginal Junction SSTs

Semen from chickens and turkeys were diluted to 800×10^6 spermatozoa/ml in 0.15M NaCl/TES. Intravaginal or uterovaginal junction (2.3.1.2.4.) insemination of hens with 20×10^6 chicken or turkey spermatozoa was then performed.

Sperm access to the uterovaginal junction SSTs was assessed as described in 2.3.1.2.4..

2.6.3. Investigation of the Physiological Basis of Oviducal Sperm Selection in the Chicken

2.6.3.1. Protocol for Immunofluorescent and Live/Dead Staining of Intravaginally Inseminated Chicken and Turkey Spermatozoa Recovered by Vaginal Lavage

Chicken hens were inseminated intravaginally with 200×10^6 chicken or turkey spermatozoa. Vaginal lavage was subsequently performed on hens 20 minutes, 1, 2, 4, 8 or 18 hours post-insemination by flushing the anterior vagina with 1 ml of 0.15M NaCl/TES into 5 ml polythene tubes. Sperm counts were performed on washings obtained 20 minutes post-insemination.

Vaginal washings were centrifuged at 700 g for 15 minutes at 5 °C and non-specific binding sites blocked with 200 µl of normal rabbit serum diluted 1:10 with 0.15M NaCl/TES, for 30 minutes at 37 °C. After centrifugation at 700 g for 10 minutes at 5 °C, spermatozoa were washed with 500 µl of 0.15M NaCl/TES and incubated with 100 µl of FITC conjugated rabbit antichickens IgG (Sigma Chemical Co.) diluted 1:30 with 0.15M NaCl/TES, for 30 minutes at 37 °C. The sperm suspension was then made up to 500 µl with 0.15M NaCl/TES, centrifuged at 700 g for 10 minutes at 5 °C, washed with 500 µl of 0.15M NaCl/TES, and resuspended in 50 µl of 0.15M NaCl/TES. Control non-inseminated spermatozoa (1×10^6) were treated identically.

Spermatozoa were examined by epifluorescence microscopy at X400 and X1000 (oil) magnification for the presence and distribution of chicken IgG on their surface.

Live/dead staining of lavaged spermatozoa was carried out using nigrosin and eosin (2.3.1.2.3.). Control non-inseminated spermatozoa diluted 1:100 with 0.15M NaCl/TES were treated identically.

2.6.3.2. Investigation of the Relationship Between Sperm Plasma membrane Permeabilisation and Immunoglobulin Binding to the Sperm Surface

2.6.3.2.1. Determination of the Comparative Efficiencies of Nigrosin and Eosin, and Propidium Iodide as Vital Stains

Chicken semen was diluted 1:250 with 0.15M NaCl/TES. The fluorescing vital stain propidium iodide (Sigma Chemical Co.) (Harrison & Vickers, 1990; Graham et al., 1990) was diluted from stock to 400 and 40 µg/ml in 0.15M NaCl/TES. For assay 5 µl of propidium iodide (PI) solution was added to 95 µl of sperm suspension. Nigrosin and eosin was used as described in 2.3.1.2.3..

In some cases, the concentrations of spermatozoa recovered by vaginal lavage were low, particularly many hours following insemination. Consequently, several minutes

were often spent examining single microscope slides, and accordingly it was important to establish whether PI itself would influence the live/dead ratio as a function of time.

Spermatozoa were incubated with PI at both concentrations and 15 μ l of sperm suspension transferred to a microscope slide with coverslip, examined by epifluorescence microscopy at X400 magnification and the percentage of spermatozoa stained determined. The assessment was repeated after 5 and 10 minutes.

Nigrosin and eosin-stained spermatozoa (15 μ l) were smeared over a microscope slide and air-dried (Lake & Stewart, 1978). Two further 15 μ l aliquots were transferred to microscope slides and incubated under coverslips for 5 and 10 minutes. Coverslips were removed and spermatozoa smeared across the slides and air-dried. Slides were viewed microscopically at X1000 (oil) magnification and the percentage of spermatozoa stained determined.

In addition, 15 μ l of sperm suspension containing PI at a concentration of 20 μ g/ml was transferred to a microscope slide after 30 minutes incubation at room temperature and immediately assessed as described above, using epifluorescence microscopy.

2.6.3.2.2. Protocol for Antichicken IgG and Propidium Iodide Staining of Spermatozoa Recovered from the Vagina by Lavage, Following Intravaginal Insemination

Vaginal lavage was performed after intravaginal insemination of chicken spermatozoa as described in 2.6.3.1.. Following incubation with FITC-conjugated rabbit antichicken IgG (2.6.3.1), spermatozoa were resuspended in 50 μ l of 0.15M NaCl/TES containing PI at a final concentration of 2 μ g/ml. Spermatozoa were examined by epifluorescence microscopy at X400 and X1000 (oil) magnification and assessment made in terms of the percentage of spermatozoa staining dead (permeated by PI), the percentage of spermatozoa with chicken IgG bound to their surface, and the percentage of spermatozoa exhibiting both stains.

2.6.3.3. Investigation of Chicken Immunoglobulin Binding and Live/Dead Status of Spermatozoa Recovered by Vaginal Lavage, and Assessment of Sperm Access to the Newly Ovulated Egg In-Vivo, in Previously Inseminated and Virgin Hens of the Same Age

Virgin hens and hens previously inseminated a number of times over preceding months were intravaginally inseminated and vaginal lavage, immunofluorescent

staining, live/dead staining and their subsequent assessments carried out as described in 2.6.3.1..

Sperm access to the newly ovulated egg in-vivo in intravaginally inseminated hens was assessed as described in 2.3.1.2.5..

2.6.3.4. Assessment of Chicken Immunoglobulin Binding to Human and Gerbil Spermatozoa Recovered from the Chicken Vagina by Lavage

Chicken hens were inseminated intravaginally with human semen containing 50×10^6 ejaculated spermatozoa or pooled gerbil epididymal/vas deferens spermatozoa and recovered by vaginal lavage (2.6.3.1.) 10 minutes post-insemination. Lavaged spermatozoa and non-inseminated control spermatozoa (1×10^6) were stained with FITC conjugated rabbit antichickens IgG and subsequently assessed as described in 2.6.3.1..

2.6.3.5. Assessment of Chicken Immunoglobulin Binding to Chicken Spermatozoa Recovered from the Uterovaginal Junction and Infundibulum Following Intravaginal and Intrauterine Insemination

Chicken hens were intravaginally inseminated twice daily over 3 days with 1×10^9 chicken spermatozoa. Forty eight hours following final insemination, hens were sacrificed by cervical dislocation and the entire oviduct excised. Alternatively, hens were inseminated intrauterinely with 200×10^6 spermatozoa and sacrificed 3-4 hours after insemination. The uterovaginal junctions and infundibulae were removed separately, opened up and placed lumen-down into a small volume of 0.15M NaCl/TES in a petri dish. Spermatozoa were removed from the mucosa by agitation, centrifuged at 700 g for 15 minutes at 5 °C, stained with FITC-conjugated rabbit antichickens IgG and subsequently assessed as described in 2.6.3.1..

2.6.3.6. Protocol for Histological Staining of Chicken Vaginal Mucosal Smears Pre- and Post-insemination

Mucosal smears were obtained from non-inseminated hens and from hens 2 hours post-insemination. Smears were obtained by inserting a modified tongue depressor into the everted vagina and gently scraping in a clockwise direction.

Material obtained was smeared onto microscope slides and air-dried. Leishmans Stain (BDH Chemicals) (0.15% in methanol) was added, diluted with distilled water after 2 minutes, then left at room temperature for 15 minutes. Slides were then rinsed thoroughly with distilled water, air-dried and examined by light microscopy at X400 and X1000 (oil) magnification.

2.6.4. Assessment of Chicken Immunoglobulin Binding and Live/Dead Status of Chicken Spermatozoa Incubated In-Vitro with Vaginal Mucosa, and Subsequent Sperm Access to the Newly Ovulated Egg In-Vivo

Chicken hens were sacrificed by cervical dislocation and the vaginal and infundibular regions excised. Tissue was cut longitudinally and incubated at 40 °C in buffered Earle's Balanced Salt Solution with the mucosal surface at the liquid-air interface. After 10 minutes incubation, 10×10^6 chicken spermatozoa in 100 μ l of Earle's Balanced Salt Solution were layered over the mucosal surface. A similar number of control spermatozoa were incubated in the same buffer at 40 °C. After a 60 minute incubation, spermatozoa were displaced into the buffer by agitation, a live/dead count performed (2.3.1.2.3.) and the sperm concentration adjusted to 5×10^6 in 500 μ l. Sperm suspensions were centrifuged at 700 g for 15 minutes at 5 °C and immunofluorescent staining and subsequent sperm

assessment carried out on control and vaginally incubated spermatozoa as described in 2.6.3.1..

Spermatozoa intended for intravaginal insemination following incubation with vaginal mucosa in-vitro were initially diluted 1:1 with Earle's Balanced Salt Solution and 200×10^6 layered over each vaginal mucosa. After recovery from the tissue, 20×10^6 control or vaginally-incubated spermatozoa in $50 \mu\text{l}$ of the same buffer were inseminated per hen, and sperm access to the newly ovulated egg assessed by counting the number of spermatozoa trapped in the outer perivitelline layer of the first fertile egg (2.3.1.2.5.).

2. 7. Investigation of Sperm Surface Changes Induced
by the Oviduct

2. 7. 1. Assessment of Sperm Surface Change in
Spermatozoa Recovered from the Uterovaginal
Junction and Infundibulum at Around the Time of
Ovulation following Intravaginal Insemination

Chicken hens were inseminated intravaginally twice daily for 3 days with 1×10^9 chicken spermatozoa, and sacrificed by cervical dislocation 25 minutes following oviposition on the second day after the final insemination. Spermatozoa were recovered from uterovaginal junction and infundibular mucosae as described in 2.6.3.5.. Each sample was divided into 2, centrifuged at 700 g for 15 minutes at 5 °C and pellets resuspended in 500 µl of 0.15M NaCl/TES or 0.15M NaCl/TES containing 4% formaldehyde. After incubation at room temperature for 20 minutes, sperm suspensions were centrifuged at 700 g for 10 minutes at 5 °C, washed with 500 µl of 0.15M NaCl/TES and resuspended in 100 µl of 0.15M NaCl/TES. Control ejaculated spermatozoa (0.25×10^6) were treated similarly. Immunofluorescent staining of fixed spermatozoa and WGA staining of unfixed and fixed spermatozoa were carried out as described in 2.2.1.2. and 2.2.1.3. respectively.

Spermatozoa were examined by epifluorescence microscopy at X400 and X1000 (oil) magnification for oviduct-induced sperm surface changes.

2.7.2. Recovery of Spermatozoa from the Chicken Vagina
by Lavage, and WGA Staining, following
Intravaginal Insemination

Chicken hens were inseminated intravaginally with 200×10^6 chicken spermatozoa. Vaginal lavage was carried out after 1 hour or 5 hours, as described in 2.6.3.1.. Washings were centrifuged at 700 g for 15 minutes at 5 °C and the pellets resuspended in 45 μ l of 0.15M NaCl/TES. Lavaged and control ejaculated (1×10^6) spermatozoa were stained with WGA as described in 2.2.1.3. and examined by epifluorescence microscopy at X400 and X1000 (oil) magnification for changes in lectin staining related to residence within the vagina.

2.7.3. Investigation of the Effect of Exposure of Spermatozoa to Ovarian Pocket Fluid (OPF) and Perivitelline Layer of the Egg In-Vitro, on Sperm Surface Antigenicity and WGA Staining

2.7.3.1. Collection and Preparation of OPF, and Perivitelline Layer from Newly Ovulated, Follicular and Laid Eggs

Chicken hens were sacrificed by cervical dislocation 25 minutes following oviposition and the abdomen opened. The oviduct was manipulated until the ovarian follicles could be observed, and ovarian pocket fluid (OPF), free from blood, collected by pasteur pipette and microcentrifuged at 13000 g for 10 minutes.

Newly ovulated eggs were collected from the body cavity prior to entering the infundibulum. Squares of inner perivitelline layer approximately 1 cm² in area were excised from the animal and vegetal poles, washed thoroughly with 0.15M NaCl/TES and immersed in Minimum Essential Medium (Modified Eagle's; Flow Laboratories). Early (10-15 mm diameter) and intermediate (20-25 mm diameter) follicular oocytes were excised from the ovary, opened, washed thoroughly with 0.15M NaCl/TES to remove the yolk, and the inner perivitelline layer teased free of the overlying follicular membrane. After thorough washing to remove adherent cells, 1 cm² pieces of inner perivitelline layer were immersed in Minimum Essential

Medium (M.E.M.). Whole perivitelline layer from freshly laid eggs obtained from non-inseminated hens were prepared as described for inner perivitelline layer.

2.7.3.2. Incubation of Spermatozoa with OPF and Perivitelline Layer of the Egg In-Vitro, and Subsequent Sperm Immunofluorescent and WGA Staining

Chicken semen was diluted to 8×10^6 spermatozoa/ml of M.E.M., and 500 μ l added to 4 ml polycarbonate tubes containing 500 μ l of M.E.M. or OPF. The sperm suspensions were incubated at 40 °C for 10 minutes prior to the addition of 1 cm² sheets of inner perivitelline layer from the animal or vegetal pole of newly ovulated eggs to one tube each containing M.E.M. alone or M.E.M. + OPF. Vegetal pole from follicular eggs, or whole perivitelline layer from laid eggs were similarly treated. After a further incubation at 40 °C for 60 minutes (incubations were also carried out over the full 70 minute period in the absence of perivitelline layer), the membranes were removed, the sperm suspensions divided equally and centrifuged at 700 g for 10 minutes at 5 °C, and pellets resuspended in 500 μ l of 0.15M NaCl/TES or 0.15M NaCl/TES containing 4% formaldehyde (v/v). After a 20 minute incubation at room temperature the sperm suspensions were centrifuged at 700 g for 10 minutes at 5 °C and subsequently stained with FITC-conjugated WGA or antiserum

raised against chicken ejaculated seminal plasma proteins, and FITC-conjugated goat antirabbit IgG, as described in 2.2.1.3. and 2.2.1.2. respectively.

Spermatozoa were subsequently examined by epifluorescence microscopy at X400 and X1000 (oil) magnification for OPF and inner perivitelline layer induced changes in sperm surface antigenicity and WGA binding, and differing effects produced by the different perivitelline layers used.

2. 8. In-Vitro Investigation of Sperm-Egg
Interaction in Domestic Birds

2. 8. 1. Assessment of Inner Perivitelline Layer
Hydrolysis Resulting from In-Vitro Incubation
with Spermatozoa, and Investigation of the
Effect of OPF on Sperm-Inner Perivitelline
Layer Interaction In-Vitro

Inner perivitelline layer from the animal and vegetal poles of newly ovulated eggs, vegetal pole of follicular eggs, and whole perivitelline layer from the vegetal pole of laid eggs were prepared as described in 2. 7. 3. 1..

Ejaculated chicken semen was diluted to 40×10^6 spermatozoa/ml of M.E.M. and 500 μ l added to 500 μ l of M.E.M. or OPF. The sperm suspensions were subsequently incubated for 30 minutes at 40 °C prior to the addition of perivitelline layer. Only inner perivitelline layer from the vegetal pole of newly ovulated eggs was incubated in the presence of OPF. Motility was checked microscopically approximately one minute after addition of inner perivitelline layer, and membranes were removed after 5 minutes incubation, washed with 0.15M NaCl/TES and fixed for 2 hours in 0.15M NaCl/TES containing 8% formaldehyde (v/v). After washing with 0.15M NaCl/TES, membranes were stained overnight in 250 μ l of 0.15M NaCl/TES containing 10 μ g/ml of FITC-conjugated WGA. Control membranes not incubated with spermatozoa were identically fixed and

stained. Subsequent to staining, quantitation of points of hydrolysis on the inner perivitelline layer was carried out. Assessment was made in terms of the number of points of hydrolysis per full microscopic field at X400 magnification (0.55 mm diameter), using epifluorescence microscopy.

Parallel incubations were run, in which inner perivitelline layer sheets were incubated with spermatozoa in the presence or absence of OPF for 2 hours. Eosin exclusion (2.3.1.2.3.) was assayed immediately prior to the addition of inner perivitelline layer sheets, after a further 90 minute incubation in the absence of perivitelline layer, and after 5 minutes and 90 minutes incubation with inner perivitelline layer from the vegetal pole of newly ovulated eggs. Motility (2.3.1.2.1.) was assayed after 10 minutes incubation in the presence or absence of inner perivitelline layer, following the initial 30 minute incubation.

2.8.2. In-vitro Investigation of the Specificity of Sperm-inner Perivitelline Layer Interaction

2.8.2.1. SDS-PAGE of Perivitelline Layer from the Animal and Vegetal Poles of Freshly Laid Eggs

Twelve freshly laid eggs from non-inseminated hens were opened and the ovum and albumen separated. The animal

pole was identified and 1 cm² pieces of perivitelline layer from both poles excised and washed thoroughly with 0.15M NaCl/TES. Membranes were subsequently immersed in 3 ml of 0.15M NaCl/TES and sonicated (DAWE SONIPROBE 7530A).

Molecular weight markers and membrane preparations were diluted to a final concentration of 1:20 and 1:1 respectively in boiling mix, subjected to electrophoresis and silver stained (2.2.2.2.).

Electrophoretic profiles were subsequently examined for differences in protein composition between the animal and vegetal poles.

2.8.2.2. In-Vitro Assessment of Heterologous Avian Sperm-inner Perivitelline Layer Interaction

Newly ovulated chicken and guinea fowl eggs were collected and the inner perivitelline layer punctured and washed free of yolk proteins with 0.15M NaCl/TES. Incubation of ejaculated chicken, turkey, guinea fowl and duck spermatozoa with inner perivitelline layer from the vegetal pole of chicken and guinea fowl eggs in the absence of OPF, and subsequent assessment of sperm-inner perivitelline layer interaction were carried out as described in 2.8.1..

2.8.2.3. Assessment of the Effect of Preincubation of Chicken and Turkey Spermatozoa with Sonicated Chicken Inner Perivitelline Layer, and Chicken Spermatozoa with Sonicated Chicken, Quail and Turkey Perivitelline Layer from Laid Eggs, on Subsequent Sperm-Chicken Inner Perivitelline Layer Interaction In-Vitro

Inner perivitelline layer was obtained from 4 newly ovulated chicken eggs after puncturing and washing free of yolk proteins with 0.15M NaCl/TES, immersed in 3 ml of M. E. M. and sonicated (2.8.2.1.). Whole perivitelline layer was obtained in the same way from 4 chicken, 4 turkey and 8 quail laid eggs. The resultant homogeneous suspensions were microcentrifuged at 13000 g for 10 minutes and the supernatant protein concentrations determined spectrophotometrically (CECIL CE 373 SPECTROPHOTOMETER) at 280 nm, from a bovine serum albumin (fraction V; Sigma Chemical Co.) calibration curve.

Chicken semen was diluted to 20×10^6 spermatozoa in 500 ul of sonicated preparation or M. E. M. alone in 4 ml polycarbonate tubes and the volume made up to 1 ml with M. E. M.. Turkey spermatozoa were diluted only in the sonicated preparation of chicken inner perivitelline layer or in M. E. M. alone. After incubation at 40 °C for 60 minutes, 1 cm² pieces of inner perivitelline layer were added to each tube, and subsequently removed after 5 minutes incubation at 40 °C. Sperm motility was checked

microscopically approximately one minute after addition of membrane.

Assessment of sperm-inner perivitelline layer interaction was carried out as described in 2.8.1..

2.8.2.4. SDS-PAGE of Sonicated Chicken Inner Perivitelline Layer and Chicken, Turkey and Quail Perivitelline Layer from Laid Eggs, following Absorption with Chicken Spermatozoa

Chicken semen was diluted to 2×10^9 spermatozoa in 5 ml of 0.15M NaCl/TES, centrifuged at 1500 g for 5 minutes at 5 °C, washed with 5 ml of 0.15M NaCl/TES and resuspended in 400 µl of sonicated chicken inner perivitelline layer or chicken, turkey or quail whole perivitelline layer preparation (2.8.2.3.). After incubation at 40 °C for 2 hours with periodic gentle agitation in 4 ml polycarbonate tubes, sperm suspensions were centrifuged at 700 g for 15 minutes at 5 °C and the supernatants microcentrifuged at 13000 g for 10 minutes, the resultant supernatant being retained.

Molecular weight markers and absorbed and non-absorbed perivitelline layer preparations were diluted to final concentrations of 1:20, 10:1 and 10:1 respectively, in boiling mix. Electrophoresis and silver staining were carried out as described in 2.2.2.2., and gels were

subsequently examined for protein band absences or changes in staining intensities related to exposure to spermatozoa.

2. 9. Investigation of Differences in Sperm Surface Characteristics and Seminal Plasma Composition Among Closely and More Distantly Related Avian Species

2. 9. 1. Assessment of Interspecies Differences in Sperm Antigenicity and Presenting Carbohydrate Residues

Pooled ejaculated semen was obtained from chickens, turkeys and quail. Pooled semen was obtained from the posterior ductus deferens of ducks and pigeons as described in 2.2.1.2..

Spermatozoa from all species studied were prepared and stained with antiserum raised in rabbit against chicken seminal plasma proteins, and FITC-conjugated goat antirabbit IgG, as described in 2.2.1.2..

Stock solutions of WGA, ConA and lectin from Solanum tuberosum were as described in 2.2.1.3., with the addition of lectin from Tetragonolobus purpureas, which was 1 mg/ml in 0.15M NaCl/TES, pH 7.4 (see Appendix 1 for lectin specificities).

Spermatozoa were prepared and stained as described in 2.2.1.3., and subsequently examined by epifluorescence microscopy at X400 and X1000 (oil) magnification for

species-specific differences in staining patterns and intensities.

2.9.2. SDS-PAGE and Western Blotting of Seminal Plasmas from Different Avian Species

Semen was collected as described in 2.9.1., centrifuged at 700 g for 15 minutes at 5 °C and the supernatants microcentrifuged at 13000 g for 10 minutes and stored at -20 °C prior to electrophoresis.

Molecular weight markers and chicken, turkey, quail, pigeon and duck seminal plasmas were diluted to final concentrations of 1:20, 1:20, 1:40, 1:10, 1:10 and 1:10 respectively in boiling mix. Electrophoresis and silver staining were carried out as described in 2.2.2.2.. Western blotting and subsequent immunological staining were carried out as described in 2.2.2.3..

Gels were subsequently examined for interspecies similarities and differences in banding patterns and intensities. Blots were examined for cross-reactivity of the antiserum raised against ejaculated chicken seminal plasma proteins with seminal plasma proteins from other species.

2.10. Statistical Analysis

Standard error of the mean (s.e.m.) values were calculated and data were compared using Student's t-test and one-way analysis of variance. Percentages form a binomial rather than a normal distribution (Zar, 1984), and were therefore transformed to arcsine values prior to calculation of s.e.m. values and subsequent statistical analysis.

CHAPTER 3. RESULTS

3. RESULTS

3. 1. Post-testicular Maturation of the Sperm Surface

3. 1. 1. Assay of Antiserum Titre by Sperm Agglutination

Rabbit antiserum raised against chicken seminal plasma proteins exhibited strong sperm-agglutinating activity at 1:10 dilution (see 2.2.1.1. for protocol). Weak agglutinating activity was also observed at 1:100 dilution, although the sperm suspension retained a degree of turbidity. Control rabbit serum exhibited no sperm agglutinating activity. The antiserum was subsequently used at 1:10 dilution for immunofluorescence and 1:100 dilution for immunological staining of Western blots.

3. 1. 2. Immunofluorescent Staining of Spermatozoa from the Ejaculate and Different Areas of the Male Reproductive Tract with Antiserum Raised Against Chicken Ejaculated Seminal Plasma Proteins

Testicular spermatozoa (mature forms) were generally unstained (see 2.2.1.2. for protocol), although a small percentage exhibited faint patchy staining, mainly restricted to the head region (Table 1, Figure 3).

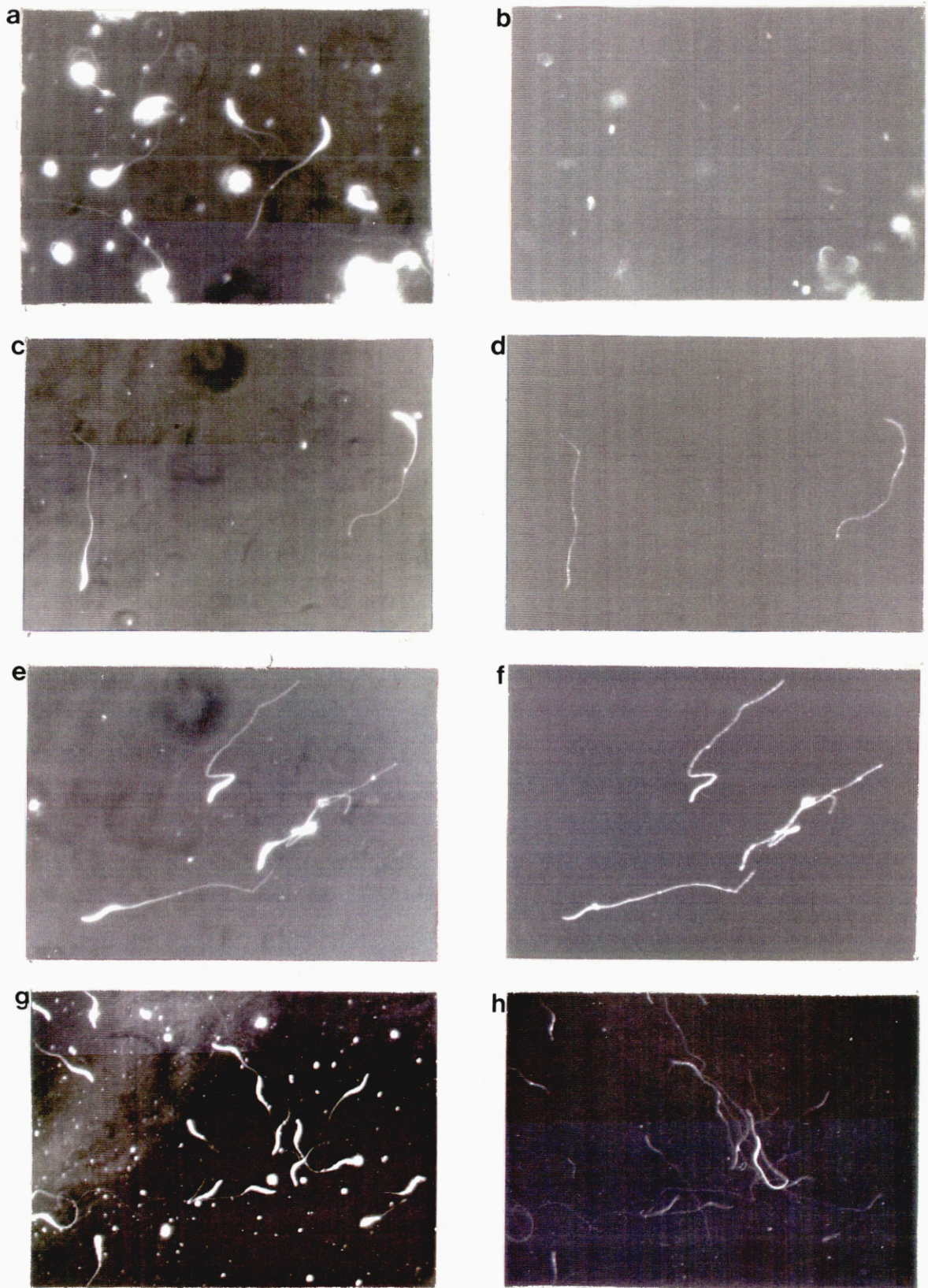


Figure 3. Photomicrographs of spermatozoa from the testes(a,b), anterior ductus deferens(c,d) and ejaculate(e,f,g,h), taken using phase contrast(a,c,e,g) and epifluorescence(b,d,f,h) microscopy. Spermatozoa were stained with rabbit antiserum raised against chicken ejaculated seminal plasma proteins, and FITC conjugated goat antirabbit IgG. A single spermatozoon exhibits enhanced fluorescence(h). X400.

Ejaculated spermatozoa and spermatozoa obtained from the posterior and anterior ductus deferens showed similar affinity for antiserum raised against chicken ejaculated seminal plasma proteins, exhibiting uniform fluorescence along their entire length, although a degree of variability in the intensity and completeness of staining was evident within samples. Staining intensity generally increased between the anterior and posterior ductus deferens, and between the posterior ductus deferens and ejaculate. A small percentage of spermatozoa, not significantly different ($P>0.1$) in samples obtained from the 3 locations exhibited considerably more intense staining, generally restricted to the head and anterior flagellar regions (Table 1, Figure 3).

| Source of spermatozoa | % stained | % with enhanced staining |
|---------------------------|---------------|--------------------------|
| testes | 8.6 \pm 1.1 | 0.0 |
| anterior ductus deferens | 100.0 | 4.4 \pm 1.3 |
| posterior ductus deferens | 100.0 | 5.3 \pm 1.0 |
| ejaculate | 100.0 | 5.8 \pm 1.3 |

Table 1. Immunofluorescent staining of ejaculated chicken spermatozoa and spermatozoa obtained from the male reproductive tract, following exposure to antiserum raised against chicken ejaculated seminal plasma proteins (1:10), and FITC-conjugated goat antirabbit IgG (1:50). Values are mean \pm s.e.m. of data from 1200 spermatozoa per region from 3 cockerels.

3.1.3. Lectin Staining of Spermatozoa from the Ejaculate and Different Areas of the Male Reproductive Tract

All testicular spermatozoa examined (mature forms) exhibited some degree of staining with FITC-conjugated WGA (see 2.2.1.3. for protocol). Fixed and unfixed spermatozoa showed similar affinity for the lectin, but staining was variable within any given sample. Most spermatozoa ($88.1 \pm 10.1\%$ of unfixed, $80.3 \pm 16.7\%$ of fixed) exhibited a variable degree of flagellar staining, which was generally sparse and patchy. Staining of the head region was similar, with $72.4 \pm 15.9\%$ of unfixed and $83.0 \pm 21.1\%$ of fixed spermatozoa stained (1200 fixed or unfixed spermatozoa examined from 3 cockerels) (Figure 4).

Spermatozoa from the posterior and anterior ductus deferens and ejaculated spermatozoa all showed the same pattern of staining. Fixed and unfixed spermatozoa generally exhibited intense, uniform staining along their entire length (Figure 4), although a relatively small percentage of non-agglutinated spermatozoa, not significantly different between samples obtained from different locations ($P > 0.05$), exhibited a patchy, incompleteness of staining, mainly at the head region (Table 2). Spermatozoa were extensively agglutinated, with $>80\%$ within any given sample involved in agglutinations ranging in size from less than 10 to several thousand spermatozoa. Sperm-sperm agglutination

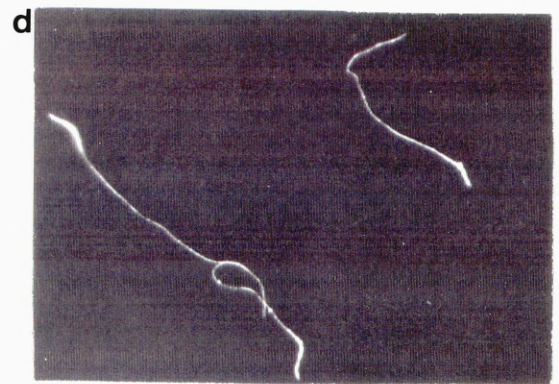
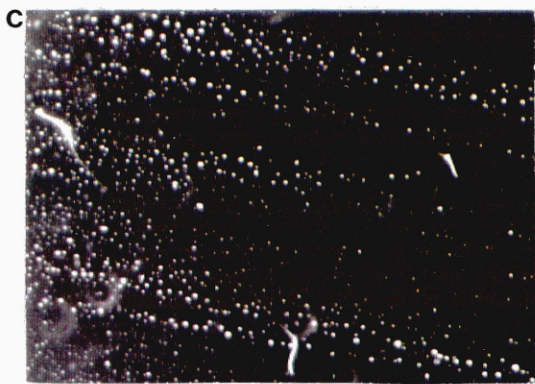
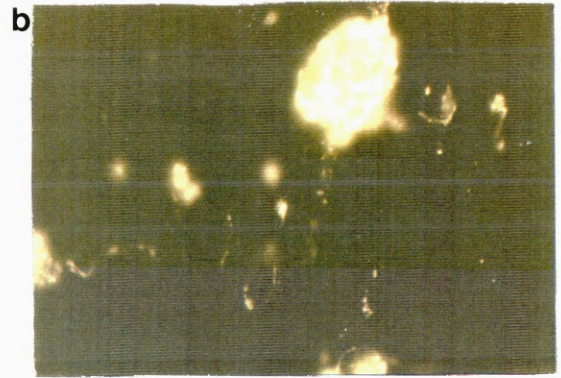
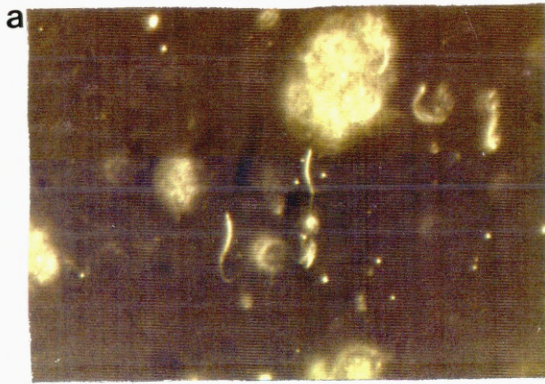


Figure 4. Photomicrographs of unfixed spermatozoa from the testes(a,b) and anterior ductus deferens(c,d,e,f) stained with FITC conjugated WGA, taken using phase contrast(a,c,e) and epifluorescence(b,d,f) microscopy.X400.

appeared to be almost exclusively flagellar, and spermatozoa observed within agglutinations exhibited more intense staining (Figure 4).

| Source of spermatozoa | % unfixed spermatozoa with reduced staining | % fixed spermatozoa with reduced staining |
|---------------------------|---------------------------------------------|-------------------------------------------|
| anterior ductus deferens | 15.3 \pm 0.8 | 21.3 \pm 3.0 |
| posterior ductus deferens | 13.8 \pm 0.8 | 15.0 \pm 0.8 |
| ejaculate | 17.5 \pm 2.3 | 20.1 \pm 2.6 |

Table 2. WGA staining of ejaculated spermatozoa and spermatozoa from the posterior and anterior ductus deferens. Values are mean \pm s.e.m. of data from 1200 spermatozoa examined from each region, from 3 cockerels.

Staining of testicular spermatozoa with FITC conjugated ConA was similar to that observed on spermatozoa from the ductus deferens and the ejaculate. Fixed and unfixed spermatozoa were similarly stained, exhibiting weak, fairly uniform fluorescence, generally restricted to the head and midpiece regions (Figure 5).

Neither fixed nor unfixed testicular spermatozoa were stained with FITC-conjugated lectin from Solanum tuberosum. However, all fixed and unfixed ejaculated spermatozoa and spermatozoa from both regions of the ductus deferens exhibited patchy fluorescence of variable

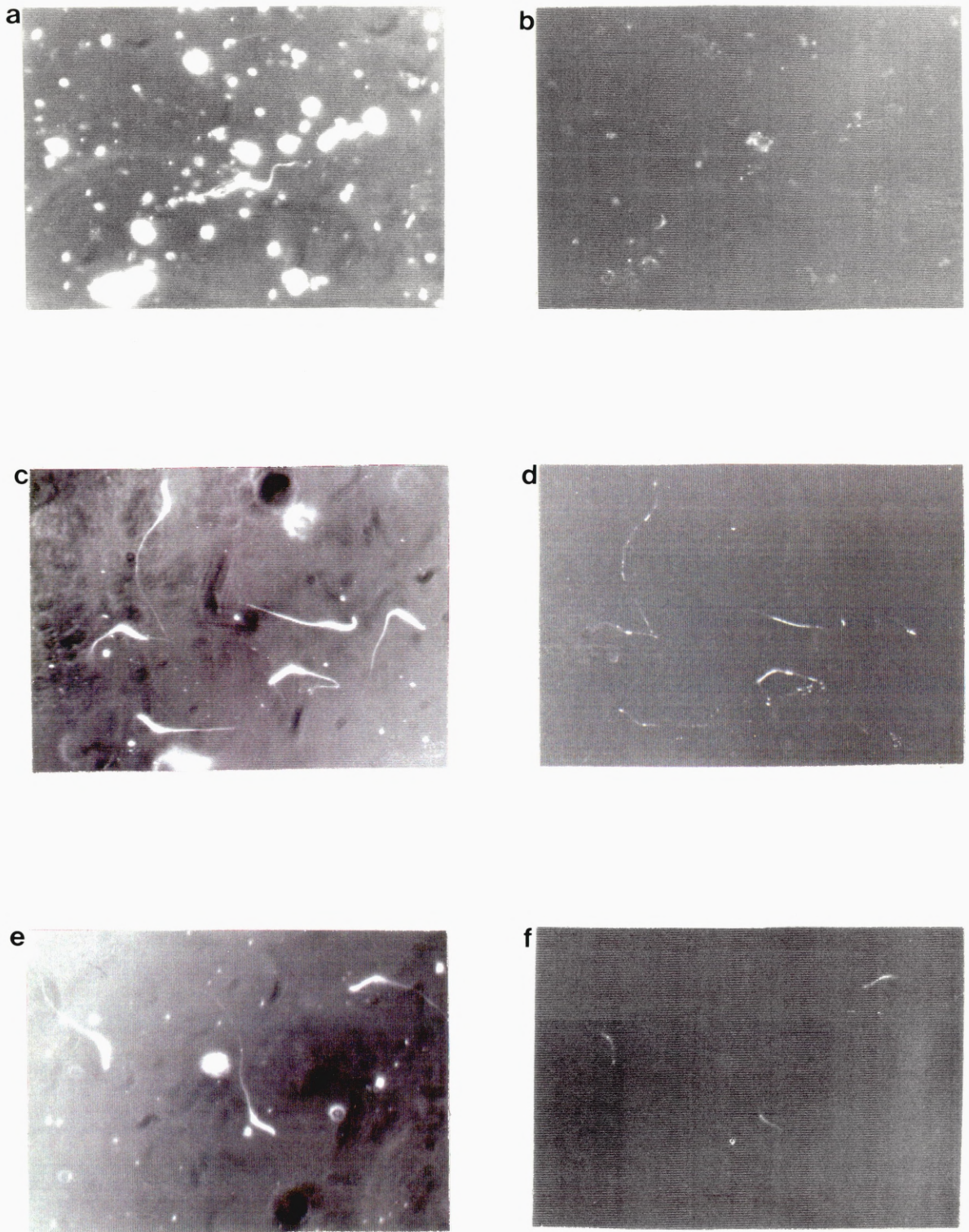


Figure 5. Photomicrographs of unfixed spermatozoa from the testes(a,b), anterior ductus deferens(c,d) and ejaculate(e,f), stained with FITC conjugated lectin from *Solanum tuberosum*(a,b,c,d) and Con A(e,f). Photographs were taken using phase contrast(a,c,e) and epifluorescence(b,d,f) microscopy.X400.

position and intensity along their entire length (Figure 5).

None of the spermatozoa tested stained with lectins from Arachis hypogea or Erythrina corallodendron.

3.1.4. Electrophoretic Analysis of Sperm Surface-Associated Proteins Extracted from Anterior and Posterior Ductus Deferens Spermatozoa with 1.0M NaCl, and of Seminal Plasmas from Anterior and Posterior Ductus Deferens, and Ejaculated Semen

3.1.4.1. Silver Stained Electrophoretic Profiles of Sperm Surface-Associated Proteins Extracted from Anterior and Posterior Ductus Deferens Spermatozoa with 1.0M NaCl, and of Seminal Plasmas from Anterior and Posterior Ductus Deferens, and Ejaculated Semen

Around 60 individual protein bands could be identified in electrophoretic profiles of ejaculated seminal plasma (see 2.2.2.2. for protocol), ranging in molecular weight from 10 kD to around 180 kD (Figure 6), with the majority of bands lying between 10 kD and 80 kD. The dominant band was at 66 kD and at seminal plasma concentrations required for examination of other prominent bands, was severely overloaded on the gel. Other prominent bands appeared at

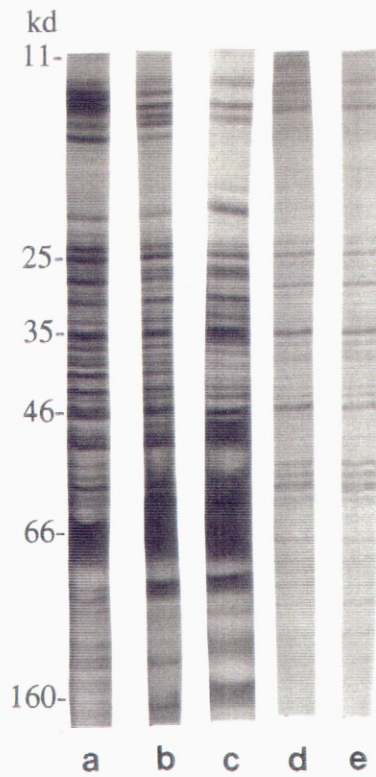


Figure 6. Silver stained electrophoretic profiles of anterior ductus deferens(a), posterior ductus deferens(b) and ejaculated(c) seminal plasmas, and sperm surface washings obtained from hypertonic buffer(1.0M NaCl) treatment of spermatozoa from the anterior(d) and posterior(e) ductus deferens.

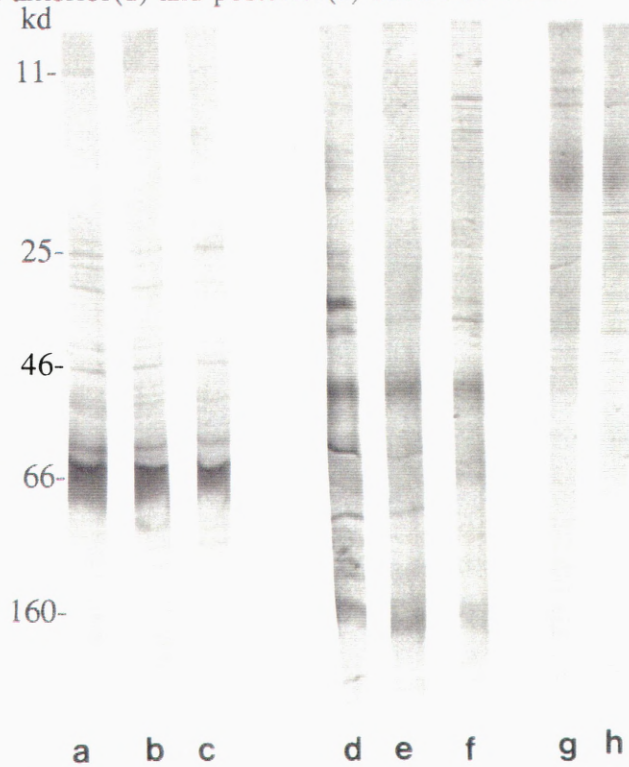


Figure 7. Western blot of ejaculated, and posterior and anterior ductus deferens seminal plasma proteins, stained with rabbit antiserum raised against chicken ejaculated seminal plasma proteins and HRP conjugated donkey antirabbit IgG(a,b,c respectively), or HRP conjugated WGA(d,e,f respectively). Lanes g and h are WGA stained sperm surface washings obtained from hypertonic buffer(1.0M NaCl) treatment of spermatozoa from the anterior and posterior ductus deferens respectively.

12, 13, 13.5, 21, 25, 27, 30, 35, 36.5, 46, 49, 51, 53, 58 and 80 kd.

A number of differences were apparent in the electrophoretic profiles of ejaculated seminal plasma and seminal plasmas from the anterior and posterior regions of the ductus deferens, relating to progression from the anterior ductus deferens to the ejaculate (Figure 6).

Protein bands at 11, 13, 24, 27, 39-40, 43 and 46 kd were more prominent in the anterior ductus deferens seminal plasma profile than in the posterior ductus deferens or ejaculated profiles. The protein band at 13 kd was intensely stained in the anterior ductus deferens profile, but considerably more weakly stained in posterior ductus deferens and ejaculated profiles, and protein bands at 12.5, 15 and 22.5 kd in the anterior ductus deferens profile were considerably more weakly stained in the posterior ductus deferens profile and absent from the ejaculated profile. Conversely, a protein of molecular weight 21 kd was observed as a prominent band in the ejaculated profile, but was more weakly stained in both ductus deferens profiles.

Some protein bands were comparably stained in ejaculated and posterior ductus deferens profiles, but were more intensely e.g. 27 kd or weakly e.g. 80 kd stained in the anterior ductus deferens profile. Other staining differences related to progression from the anterior

ductus deferens to the ejaculate included the increased staining intensity of protein bands at 49, 51 and 66 kd and the appearance of protein band complexes at around 120 and 160 kd.

The electrophoretic profiles of sperm surface washings obtained from hypertonic (1.0M NaCl) buffer treatment of spermatozoa from the anterior and posterior ductus deferens (see 2.2.2.1., 2.2.2.2. for protocol) were very similar. The main difference between the 2 profiles was a band at 11.5 kd in the anterior ductus deferens profile was absent from the posterior ductus deferens profile (Figure 6).

The relatively intensely stained protein bands at 25, 35, 46 and 60 kd in sperm surface washings profiles correspond with intensely stained bands in both seminal plasma profiles. Less intensely stained bands at 11 and 12 kd correspond with protein bands which were less intensely stained in seminal plasma profiles. However, the staining intensities of some protein bands in sperm surface washings profiles do not correspond with their staining intensities in seminal plasma. These include bands at 11.5 (anterior ductus deferens only) and 28.5 kd which are relatively intensely stained in sperm surface washings but weakly stained in seminal plasma, and bands at 12.5, 13, 21, 27, 30, 36.5, 38, 39 and 43 kd which were relatively weakly stained in sperm surface washings profiles.

Protein bands at 14, 15, 33.5, 49, 51, 53, 66 and 80 kd which were strongly stained in seminal plasma, did not appear in sperm surface washings profiles.

3.1.4.2. Antigenicity and Lectin Staining of Sperm Surface-Associated Proteins Extracted from Anterior and Posterior Ductus Deferens Spermatozoa with 1.0M NaCl, and Seminal Plasma Proteins from Ejaculated Semen and Semen from the Anterior and Posterior Ductus Deferens, following SDS-PAGE and Western Blotting

Immunological staining of Western blots of ejaculated, and anterior and posterior ductus deferens seminal plasmas with antiserum raised against chicken ejaculated seminal plasma proteins (see 2.2.2.1., 2.2.2.3. for protocol) indicates subtle changes in the antigenicities of some seminal plasma proteins, related to progression from the anterior ductus deferens to the ejaculate (Figure 7). Staining intensity generally increased between anterior ductus deferens and ejaculate.

The prominent protein band in all 3 profiles was that at 66 kd, with other intensely stained bands at 25, 27, 30, 43, 46, 49, 51, 53 and 58.5 kd.

Protein bands at 27, 30, 43, 46, 49, 51, 53, 58, 66 and 80 kd showed increased staining intensities between the

anterior ductus deferens and the ejaculate, and the protein band at 11 kd was unstained in the anterior ductus deferens profile.

Conversely, a protein band at 90 kd appeared only on the anterior ductus deferens profile.

Proteins in 1.0M NaCl sperm surface washings were generally unstained on IgG stained blots (not shown).

WGA-stained seminal plasma proteins (see 2.2.2.1., 2.2.2.4. for protocol) exhibited changes in carbohydrate character related to progression from the anterior ductus deferens to the ejaculate (Figure 7). Protein bands at 16.5, 18, 20, 25, 27, 35, 40, 49-53, 80, 120 and 160 kd could be discerned in the ejaculated seminal plasma profile, with bands at 35, 40, 49-53, 80 and 160 kd most intensely stained. Bands at 18, 20, 25, 27 and 40 kd were absent from both ductus deferens profiles and the band at 35 kd was considerably more weakly stained than in the ejaculated profile. Bands at 13, 13.5, 14 and 15 kd in the anterior ductus deferens profile were absent from the posterior ductus deferens and ejaculated profiles. An intensely stained band at 38 kd in the anterior ductus deferens profile was considerably more weakly stained in the posterior ductus deferens profile, and absent from the ejaculated profile. Seminal plasma proteins staining as glycoproteins and expressing antigenicity were observed at 25, 27, 49-53, 66 and 80 kd.

Around 20 WGA-stained protein bands were apparent in sperm surface washings obtained from hypertonic (1.0M NaCl) buffer treatment of spermatozoa from the anterior and posterior ductus deferens (Figure 7) (see 2.2.2.1., 2.2.2.4. for protocols). Bands at 11, 12.5, 13.5, 16-20, 22, and 38 kd were most intensely stained. The relatively intensely stained band at 11 kd in the anterior ductus deferens profile was absent from the posterior ductus deferens profile, and the band at 12.5 kd was considerably more weakly stained in the posterior ductus deferens profile than in the anterior ductus deferens profile. Otherwise, the ductus deferens profiles appeared similar.

3.2. Specificity of Protein Association with the Sperm Surface

Electrophoretic profiles of the soluble and particulate fractions of seminal plasma (see 2.4.1., 2.2.2.2. for protocol) indicate clear differences in protein content, although most proteins were present in both fractions (Figure 8). Most of the protein was present in the soluble fraction. The overloaded band at 66 kd in the soluble fraction was absent from the particulate fraction, as were other intensely stained bands at 33.5, 80 and 160 kd. Conversely, the 38 kd band observed in the particulate fraction was absent from the soluble fraction. This may also have been the case for bands at 35, 58 and 60 kd. Many proteins were present in relatively different concentrations between the 2 fractions e.g. bands at 25 and 27 kd were intensely stained in the particulate fraction, but appeared only as weak bands in the soluble fraction.

There were a number of similarities between the electrophoretic profiles of the particulate fraction of seminal plasma and 0.25M NaCl sperm surface washings, notably the strongly stained bands at 35, 38, 43 and 46 kd (Figure 8). There were, however, differences between the profiles. The bands at 21, 25, 27 and 36.5 kd were strongly stained in the particulate seminal plasma fraction, but considerably more weakly stained in the sperm surface washings profile.

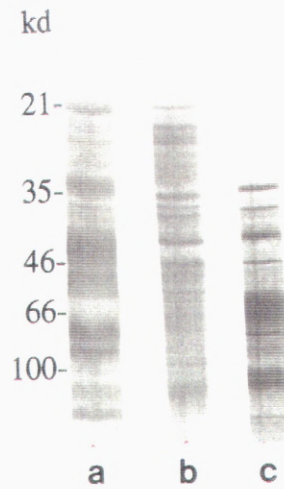


Figure 8. Silver stained electrophoretic profiles of the soluble(a) and particulate(b) fractions of chicken ejaculated seminal plasma, and sperm surface washings obtained from hypertonic buffer(0.25M NaCl) treatment of ejaculated spermatozoa(c).

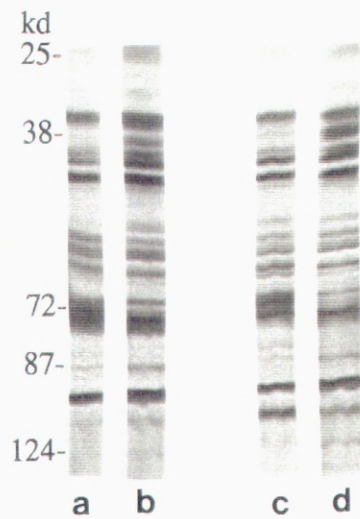


Figure 9. Silver stained electrophoretic profiles of sperm surface washings obtained from isotonic(0.15M NaCl; a,c) and subsequent hypertonic(1.0M NaCl; b,d) buffer treatment of ejaculated spermatozoa from two different pooled semen samples.

Most protein bands in the electrophoretic profiles of 0.15M and 1.0M NaCl sperm surface washings (see 2.4.2., 2.2.2.2. for protocol) were stained to a similar intensity in both cases (Figure 9). However, bands at 25, 38, 43, 87 and 124 kd were more intensely stained in 1.0M NaCl sperm surface washings profiles. Conversely, the bands at 72 and 83 kd were stained more intensely in 0.15M NaCl sperm surface washings profiles.

3. 3. Characterisation of Monospecific Polyclonal Antisera Raised Against Individual Sperm Surface-Associated Proteins Separated by SDS-PAGE

3. 3. 1. Assay of Antiserum Titre by Sperm Agglutination

| Antiserum | Lowest Sperm Agglutinating Concentration |
|-------------------|------------------------------------------|
| 11 kd protein | 1:100 |
| 25 kd protein | 1:100 |
| 43 kd protein | 1:100 |
| 46 kd protein | 1:100 |
| control rat serum | no agglutination |

Table 3. Agglutination assay results for antisera raised in rats against individual sperm surface-associated proteins.

All of the antisera produced against individual sperm surface-associated proteins (see 2.5.1., 2.5.2. for protocol) strongly agglutinated spermatozoa at 1:10 dilution, but exhibited incomplete sperm agglutinating activity at 1:100 dilution, with some degree of turbidity remaining in all sperm suspensions (Table 3). Spermatozoa viewed microscopically following exposure to antisera remained active despite extensive agglutination. Control rat serum failed to agglutinate spermatozoa.

Antisera were subsequently used at 1:100 dilution for immunological staining of Western blots, with HRP conjugated rabbit antirat IgG used at 1:200 dilution.

3.3.2. Specificity of Antisera Raised Against Individual Sperm Surface-Associated Proteins

All 4 antisera raised against individual sperm surface-associated proteins reacted with their homologous seminal plasma protein after SDS-PAGE and Western blotting (see 2.5.3., 2.2.2.2., 2.2.2.3. for protocols). However, all antisera also cross-reacted extensively with other seminal plasma proteins ranging in molecular weight from 13.5 to 80 kD. In each case, approximately 10-15 protein bands were stained, with bands at 25, 58, 66 and 80 kD stained by all antisera (see example, Figure 10). Blotted seminal plasma proteins exposed to normal rat serum and HRP-conjugated goat antirat IgG were unstained.

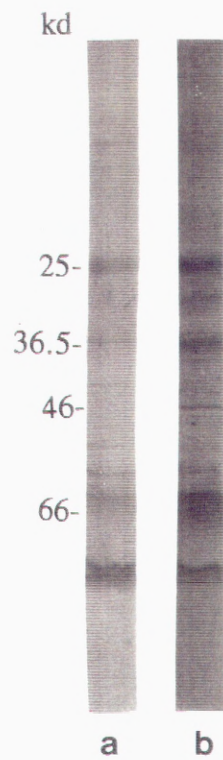


Figure 10. Western blot of chicken seminal plasma proteins following SDS-PAGE, showing cross reactivity of antisera raised in rats against the 25kd(a) and 46kd(b) sperm surface-associated proteins.

3. 4. Effect of Sperm Surface Modification on Sperm
Activity In-Vitro and In-Vivo

3. 4. 1. Effect of Hypertonic Buffer Treatment on Sperm
Function In-Vitro and In-Vivo

3. 4. 1. 1. Activity of Spermatozoa In-Vitro Following
Hypertonic Buffer Treatment

The motility, cellular ATP concentration and ability to exclude eosin (see 2.3.1.2.1. - 2.3.1.2.3. for protocols) of spermatozoa exposed to hypertonic buffer containing 0.20 and 0.25M NaCl (see 2.3.1.1. for protocol) were not significantly different from values obtained from a control incubation with buffer containing 0.15M NaCl ($P>0.1$) (figures 11-13). Furthermore, the cellular ATP concentrations did not differ significantly after a prolonged 80 minute incubation ($P>0.1$) (Table 4). The cellular ATP concentrations of spermatozoa exposed to buffer containing 0.30, 0.35 and 0.40M NaCl were not significantly different from control values after 20 minutes or 80 minutes incubation ($P>0.1$). However, sperm motility and the ability to exclude eosin differed significantly from control values ($P<0.01$).

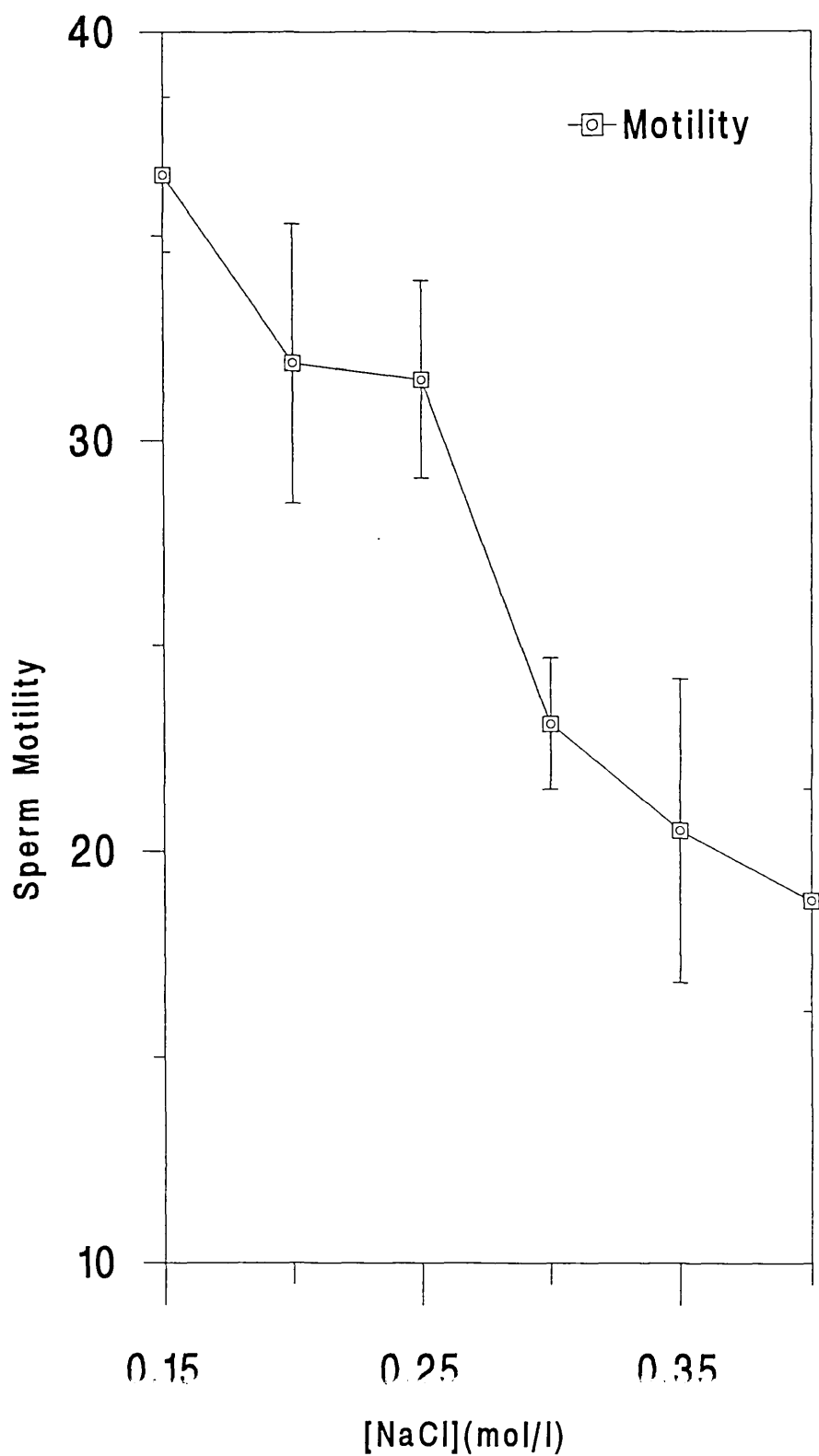


Figure 11. Effect of removal of sperm surface associated proteins on sperm motility(% Δ OD)m) in-vitro. Values are mean \pm -s.e.m. from 6 experiments.

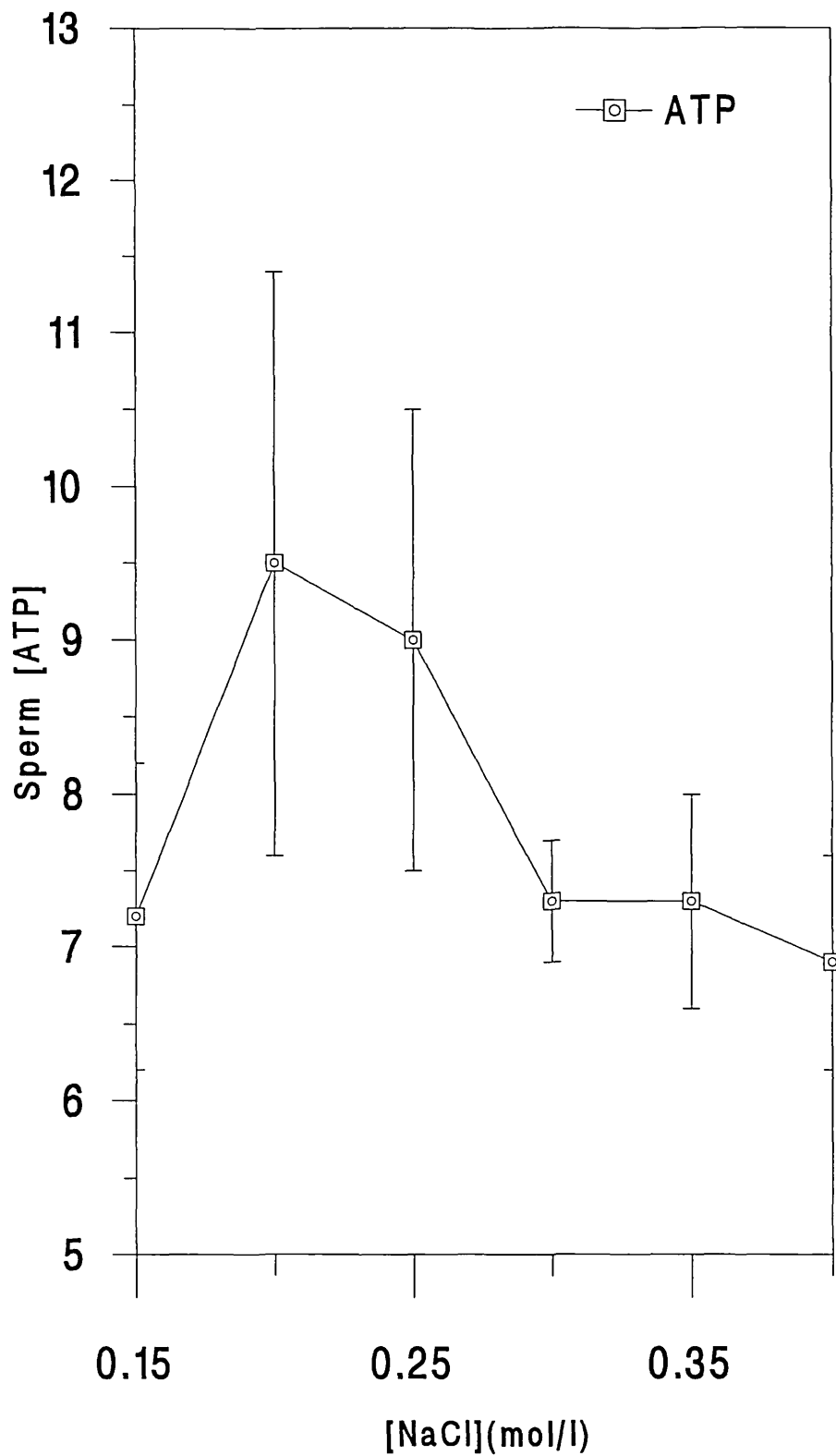


Figure 12. Effect of removal of sperm surface associated proteins on sperm[ATP](nmol/10⁹ sperm) in-vitro. Values are mean \pm s.e.m. from 6 experiments.

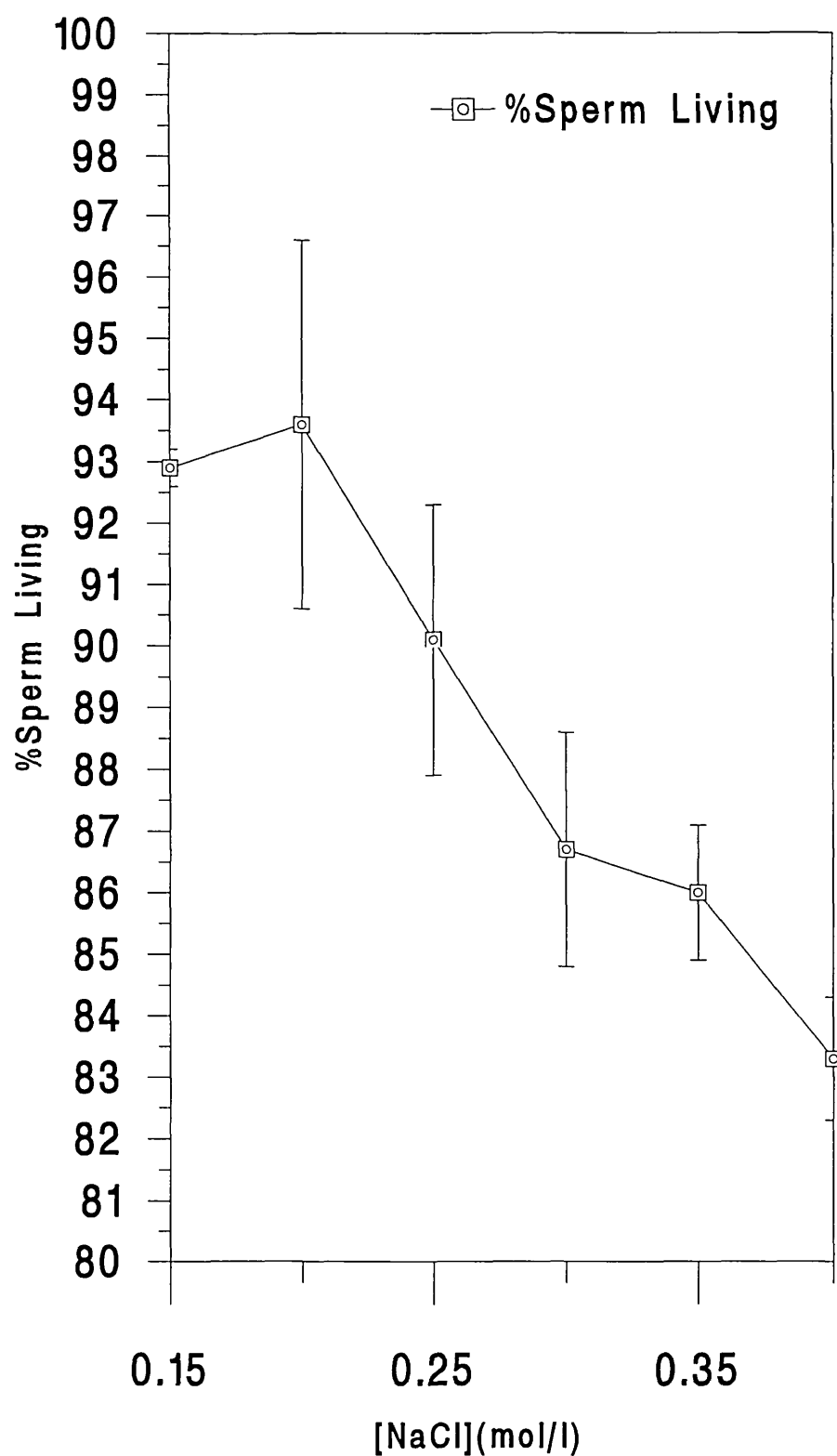


Figure 13. Effect of removal of sperm surface associated proteins on sperm live/dead status in-vitro. Values are mean \pm s.e.m. from 6 experiments.

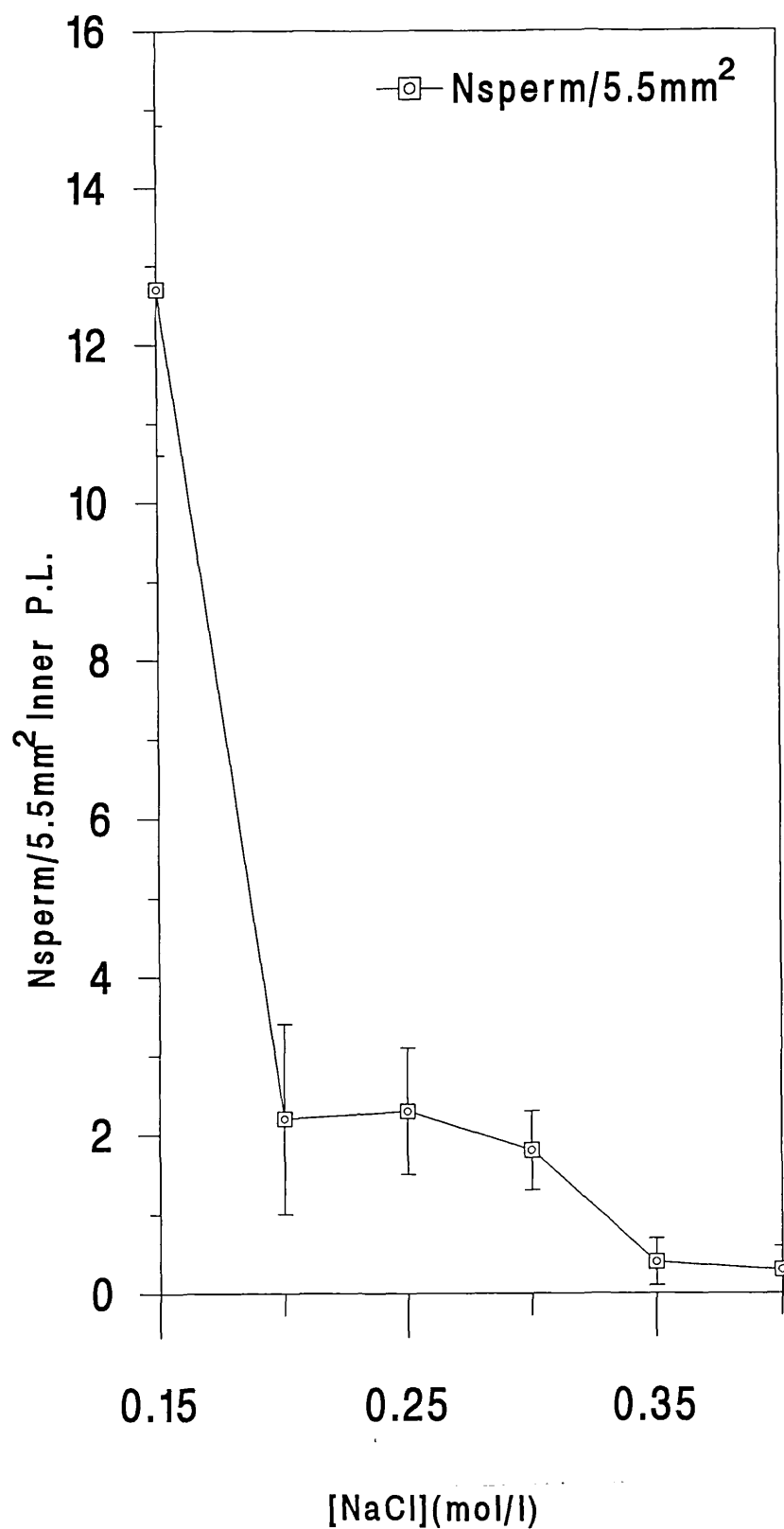


Figure 14. Effect of removal of sperm surface associated proteins on sperm access to the newly ovulated egg in-vivo. Values are mean \pm s.e.m. number of sperm per 5.5mm² area of perivitelline layer, from 6 experiments.

| Incubation time (min) | Buffer NaCl molarity | | | | | |
|--------------------------|----------------------|---------|---------|---------|---------|---------|
| | 0.15 | 0.20 | 0.25 | 0.30 | 0.35 | 0.40 |
| 20 | 7.2±1.0 | 9.5±1.9 | 9.0±1.5 | 7.3±0.4 | 7.3±0.7 | 6.9±0.7 |
| 80 | 6.5±1.2 | 6.5±1.8 | 6.4±0.9 | 5.8±0.4 | 6.5±0.9 | 5.4±0.6 |

Table 4. Cellular ATP concentrations (nmol ATP/10⁹ spermatozoa) of chicken spermatozoa during prolonged aerobic incubation at 40 °C after 15 minutes exposure to hypertonic buffers. Values are mean ± s.e.m. of data from 6 experiments.

3.4.1.2. Electrophoretic Profiles of Sperm Surface Washings Obtained by Hypertonic Buffer Treatment of Spermatozoa

Around 25 individual protein bands were identified on the electrophoretic profiles of 0.15 and 0.25M NaCl sperm surface washings (see 2.3.1.3., 2.2.2.2. for protocol). The relative staining intensities of these bands differed from their relative staining intensities in seminal plasma (Figure 15), with that of the prominent band at 66 kD in seminal plasma drastically reduced. Other intensely stained bands in seminal plasma at 15, 80, 120 and 160 kD were absent from sperm surface washings profiles. The banding patterns on sperm surface washings profiles were identical for both NaCl concentrations, although proteins in washings obtained from 0.25M NaCl treatment showed some increases in staining intensity (Figures 15, 16). Banding patterns observed were also similar to those of washings obtained from 1.0M NaCl treatment of spermatozoa from the

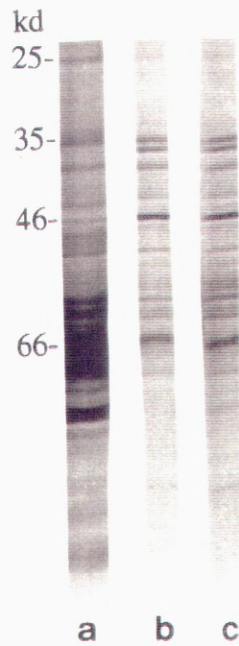


Figure 15. Silver stained electrophoretic profiles of ejaculated seminal plasma(a), and sperm surface washings obtained from isotonic(0.15M NaCl; b) and hypertonic(0.25M NaCl; c) buffer treatment of ejaculated spermatozoa.

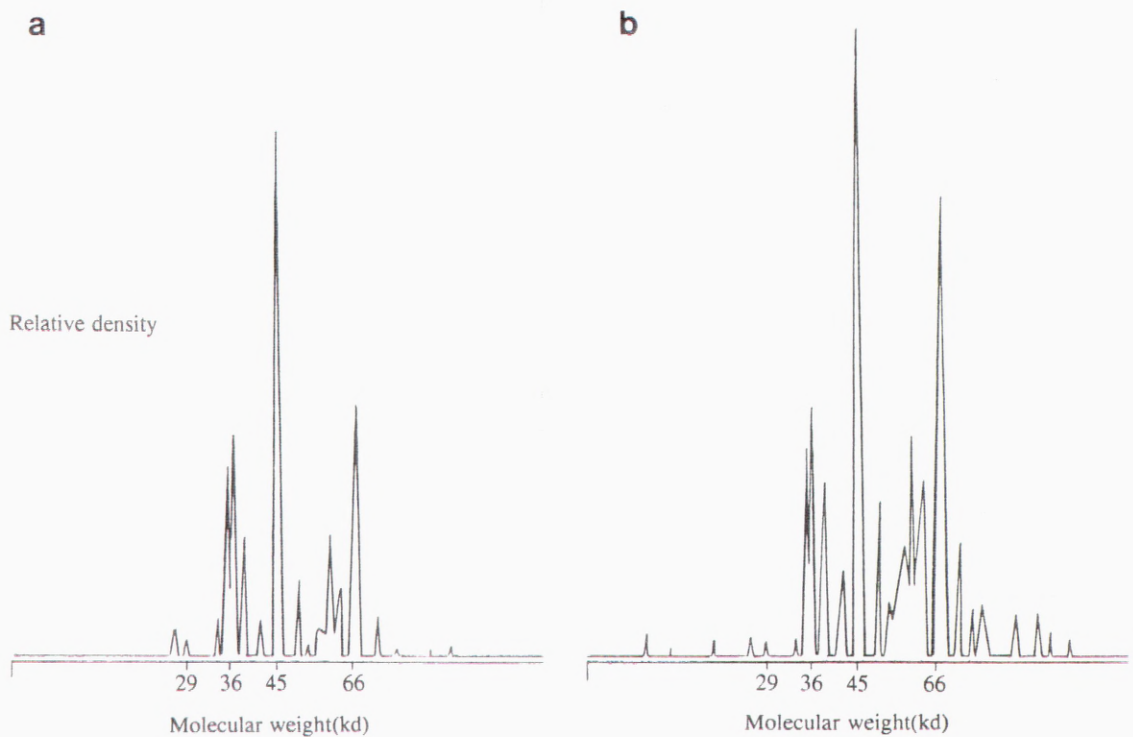


Figure 16. Densitometric scan of silver stained electrophoretic profiles of sperm surface washings obtained from isotonic(0.15M NaCl; a) and hypertonic(0.25M NaCl; b) buffer treatment of ejaculated spermatozoa.

anterior and posterior ductus deferens (Figures 6, 15). In both cases, prominent protein bands were observed at 35, 46, 58 and 60 kd. However, the bands at 35 and 36 kd were stained to a similar intensity in the sperm surface washings profile obtained from 0.15 and 0.25M NaCl treatment of ejaculated spermatozoa (Figure 15), whereas the band at 35 kd was stained considerably more intensely in sperm surface washings profiles obtained from 1.0M NaCl treatment of ductus deferens spermatozoa (Figure 6). Bands at 25 and 28.5 kd were also stained more intensely in the sperm surface washings profile obtained from 1.0M NaCl treatment. Conversely, the band at 53 kd was stained more intensely in the sperm surface washings profiles obtained from hypertonic buffer treatment of ejaculated spermatozoa.

3. 4. 1. 3. Activity of Spermatozoa In-Vivo Following Hypertonic Buffer Treatment

The ability of intravaginally inseminated spermatozoa to reach the distal infundibulum and become entrapped within the egg's outer perivitelline layer (see 2.3.1.2.5. for protocol) was significantly reduced in spermatozoa exposed to 0.20, 0.25, 0.30, 0.35 and 0.40M NaCl ($P < 0.001$), compared with control spermatozoa exposed to isotonic buffer (Figure 14).

A further assessment of the limitation to transport of spermatozoa in the hen's reproductive tract was made by examining the access of spermatozoa treated with buffer containing 0.15M or 0.25M NaCl to the uterovaginal junction SSTs in-vivo (see 2.3.1.2.4. for protocol). Following normal intravaginal insemination of spermatozoa exposed to 0.25M NaCl, only $0.5 \pm 0.2\%$ of a random selection of SSTs obtained from the anterior uterovaginal junction contained spermatozoa (mean \pm s.e.m. of data from 3 experiments, with an average of 109 SSTs examined per experiment); whereas the same sample inseminated into the uterovaginal junction lumen resulted in $35.4 \pm 1.8\%$ of SSTs containing spermatozoa (mean \pm s.e.m. of data from 3 experiments, with an average of 96 SSTs examined per experiment) (Figure 17). The percentages of SSTs containing control spermatozoa after identical inseminations were $56.0 \pm 6.3\%$ and $41.0 \pm 1.8\%$ respectively (mean \pm s.e.m. of data from 3 experiments, with an average of 101 and 104 SSTs examined per experiment respectively). The percentages of SSTs containing spermatozoa treated with 0.15 and 0.25M NaCl were not significantly different after insemination into the uterovaginal junction ($P>0.1$).

Spermatozoa reincubated with buffered seminal plasma after exposure to 0.25M NaCl/TES did not exhibit any significant change in access to the newly ovulated egg after intravaginal insemination (see 2.3.1.4., 2.3.1.2.5. for protocol), compared with control spermatozoa reincubated

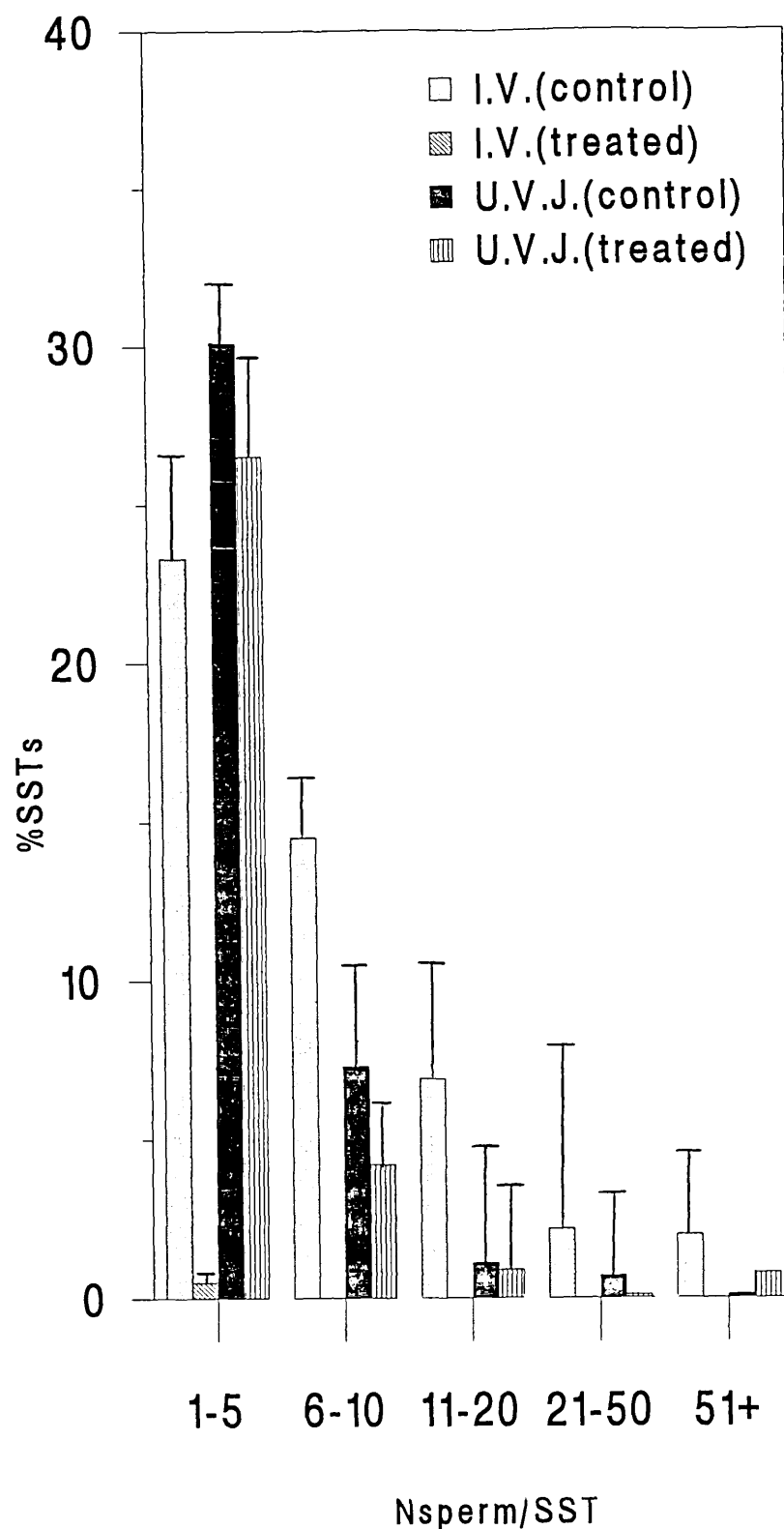


Figure 17. Effect of removal of sperm surface associated proteins on sperm access to the uterovaginal junction SSTs in-vivo. Control or treated(0.25M NaCl) spermatozoa were inseminated intravaginally or directly into the uterovaginal junction. Values are mean \pm s.e.m. from 3 experiments.

with 0.15M NaCl/TES following exposure to hypertonic buffer ($p>0.1$) (Table 5).

| Inseminated Spermatozoa | No. sperm/5.5 mm ² of perivitelline layer |
|----------------------------------------------------------------|------------------------------------------------------|
| Controls (0.15M NaCl treated) | 11.2±1.5 |
| 0.25M NaCl treated then incubated with buffered seminal plasma | 2.2±0.5 |
| 0.25M NaCl treated, then incubated with 0.15M NaCl/TES | 2.6±0.7 |

Table 5. Access of intravaginally inseminated spermatozoa to the outer perivitelline layer of eggs laid approximately 40 hours after insemination. After 0.25M NaCl treatment, spermatozoa were incubated with fresh seminal plasma diluted 1:1 with 0.15M NaCl/TES, or 0.15M NaCl/TES alone. Values are mean \pm s.e.m. of data obtained from 3 (control), 10 (0.25M NaCl treated controls) or 16 (buffered seminal plasma incubation) eggs, from 6 assessments per egg.

3.4.2. Effect of Centrifugation and Washing on Sperm Function In-Vitro and In-Vivo

3.4.2.1. Activity of Centrifuged and Washed Spermatozoa In-Vitro

Motility, cellular ATP concentration and ability to exclude eosin (see 2.3.2.2., 2.3.1.2.1. - 2.3.1.2.3. for protocols) of chicken spermatozoa in semen diluted with 0.15M NaCl/TES; semen diluted, centrifuged at 1500 g for 5 minutes and reconstituted in the same supernatant; and spermatozoa washed free of seminal plasma (see 2.3.2.1. for protocol), were not significantly different ($P>0.1$) (Figures 18-20). Furthermore, cellular ATP concentrations of the 3 samples after a prolonged 80 minute aerobic incubation were not significantly different from each other ($P>0.1$) (Table 6).

| Incubation time (min) | Semen diluted in 0.15M NaCl/TES | Semen diluted, centrifuged and reconstituted | Semen diluted, centrifuged and reconstituted in buffer |
|-----------------------|---------------------------------|----------------------------------------------|--------------------------------------------------------|
| 20 | 8.0 \pm 0.8 | 8.6 \pm 0.3 | 7.9 \pm 0.8 |
| 80 | 10.6 \pm 1.2 | 9.7 \pm 0.7 | 8.0 \pm 1.6 |

Table 6. Effect of centrifugation and washing on chicken sperm cellular ATP concentrations in-vitro. Values are mean \pm s.e.m. of data from 3 experiments, obtained after 20 minutes and 80 minutes of aerobic incubation at 40 °C following semen treatment.

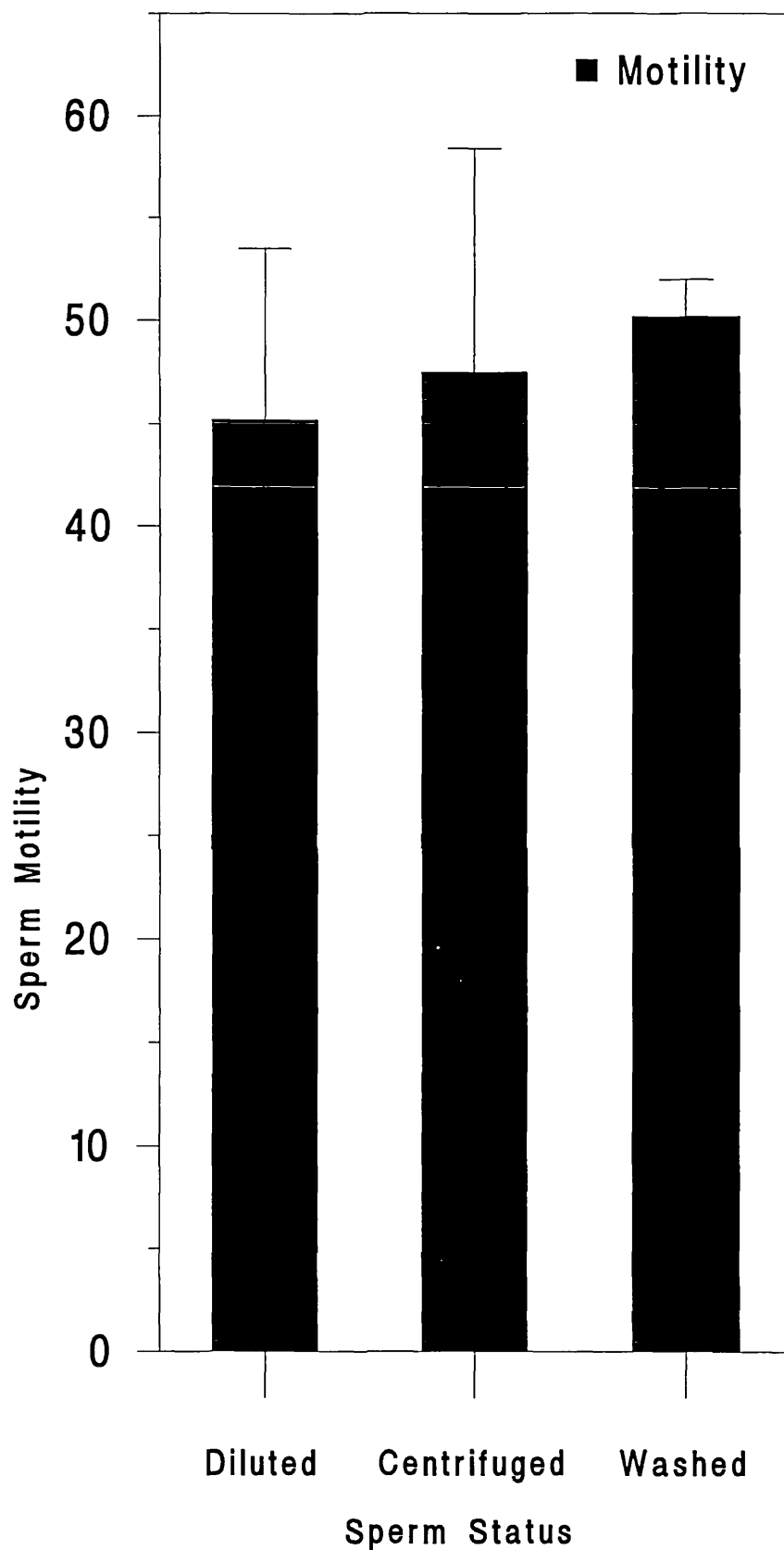


Figure 18. Effect of centrifugation and washing on sperm motility(% $[\Delta OD]_m$) in-vitro. Values are mean \pm s.e.m. from 3 experiments.

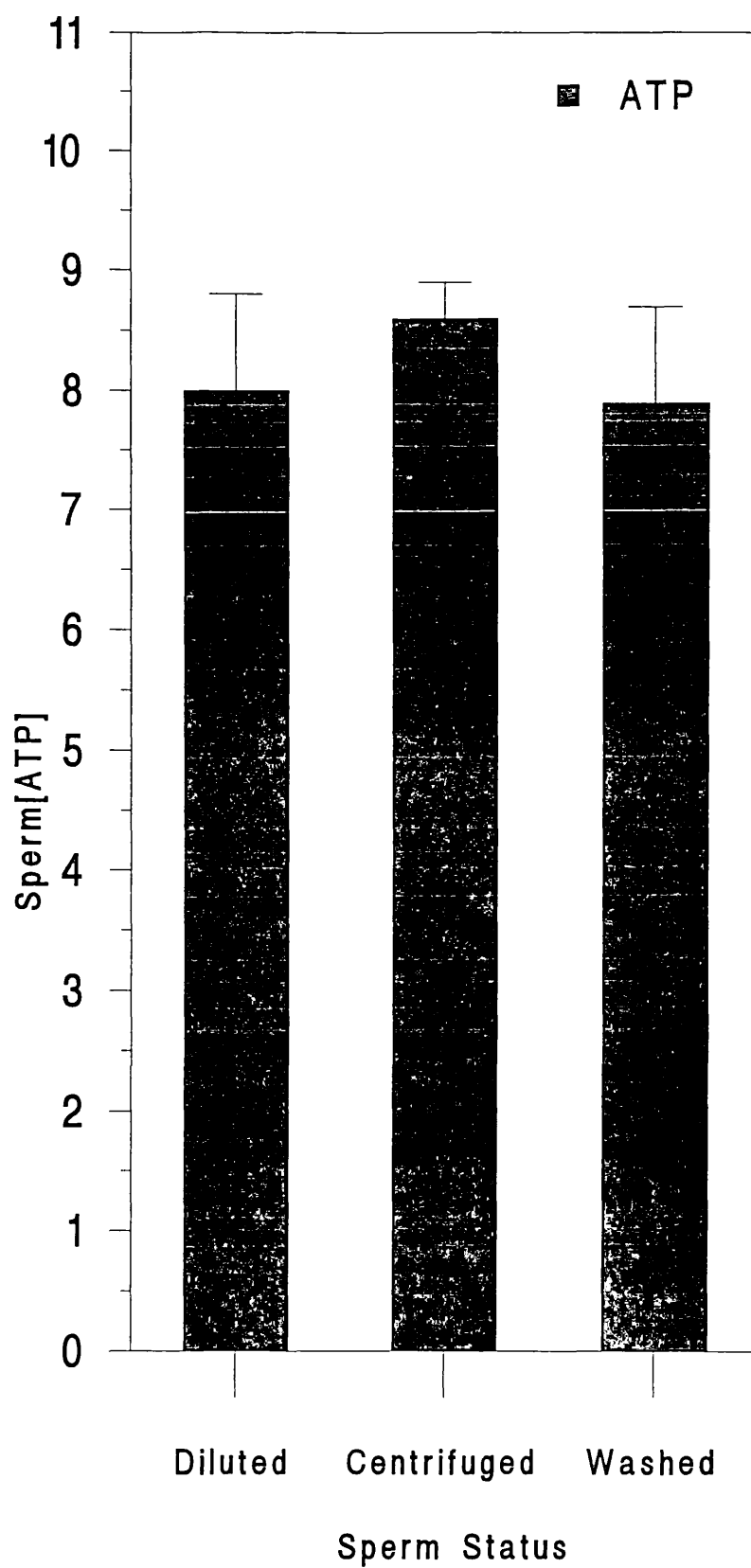
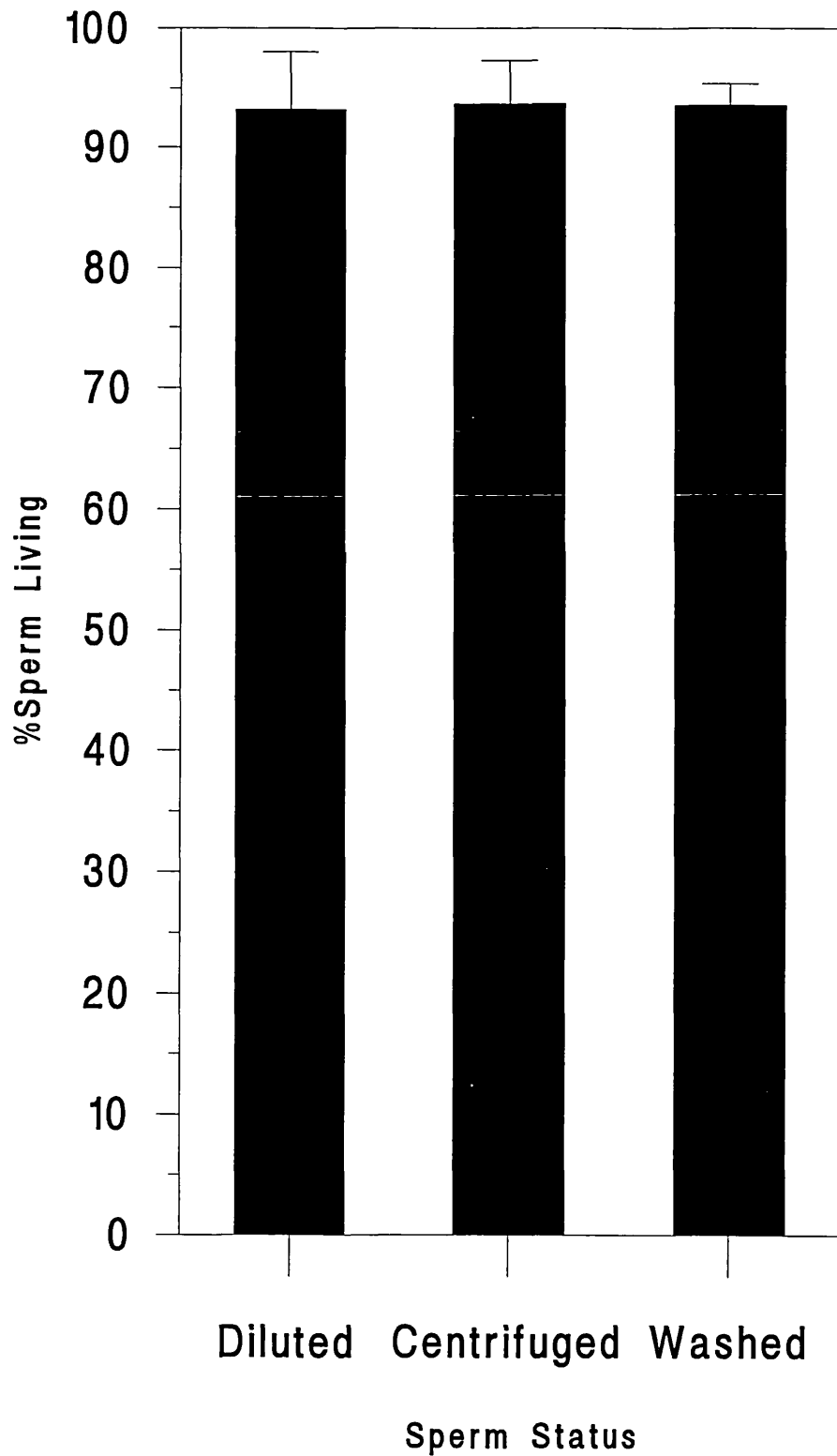


Figure 19. Effect of centrifugation and washing on sperm[ATP](nmol/10⁹sperm) in-vitro. Values are mean+/-s.e.m. from 3 experiments.



■ %Sperm Living

Figure 20. Effect of centrifugation and washing on sperm live/dead status in-vitro. Values are mean \pm s.e.m. from 3 experiments.

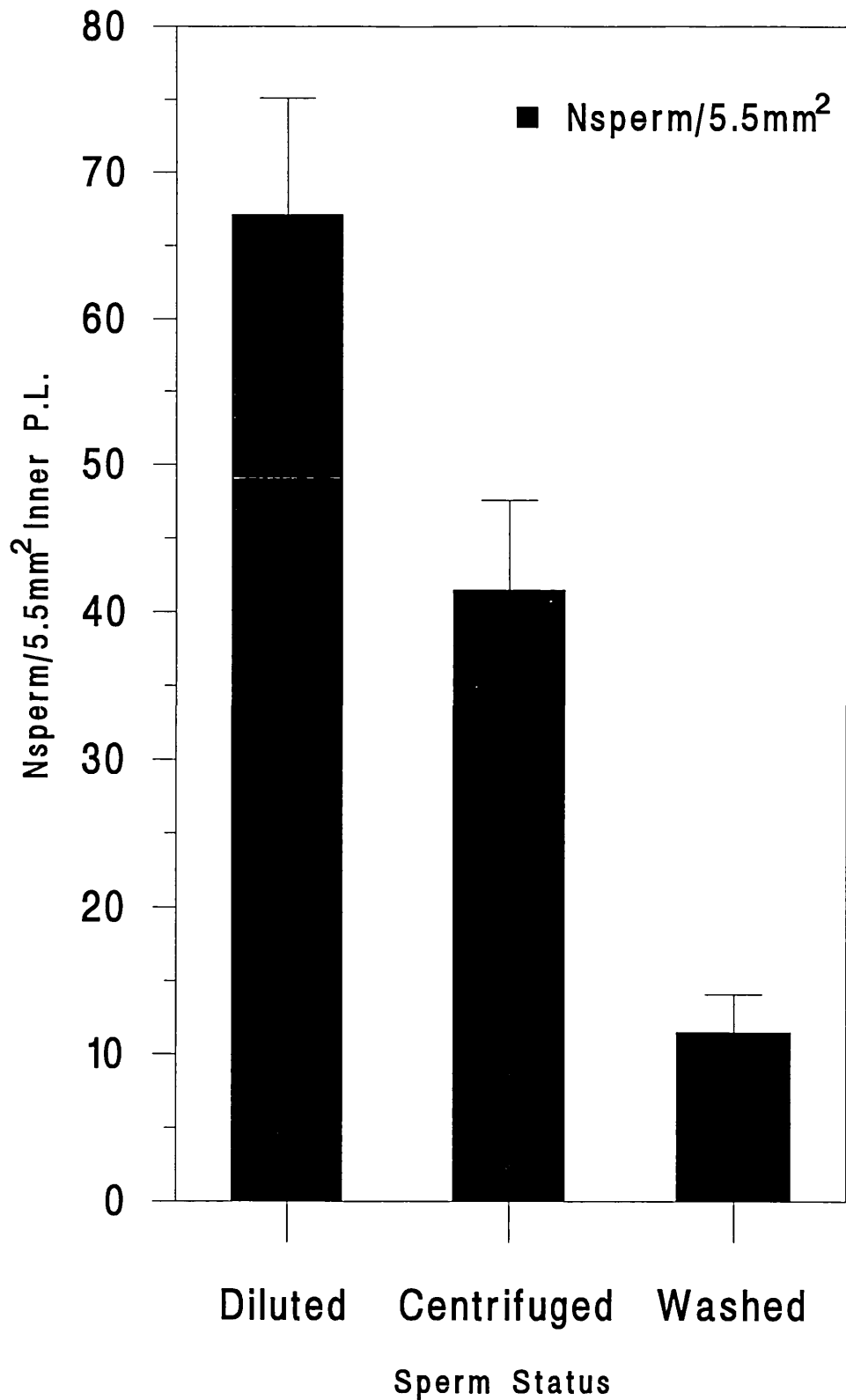


Figure 21. Effect of centrifugation and washing on sperm access to the newly ovulated egg in-vivo. Values are mean \pm s.e.m. from 19(washed or centrifuged spermatozoa) or 28(diluted only spermatozoa) eggs.

3.4.2.2. Activity of Centrifuged and Washed Spermatozoa
In-Vivo

Semen dilution, centrifugation and reconstitution in the same supernatant resulted in a significant ($P < 0.02$) 38 percentage point reduction in the ability of spermatozoa to reach the site of fertilisation (see 2.3.2.2., 2.3.1.2.5. for protocol), compared with semen dilution alone (Figure 21). Centrifugation with reconstitution of spermatozoa in 0.15M NaCl/TES reduced sperm access to the infundibulum by 83 percentage points to give a figure significantly different from both diluted and diluted/centrifuged samples ($P < 0.001$).

3.4.3. Effect of Neuraminidase Treatment on Sperm Function In-Vitro and In-Vivo

3.4.3.1. Staining of Spermatozoa with lectin from Limulus Polyphemus

Both fixed and unfixed spermatozoa incubated with FITC-conjugated lectin from Limulus polyphemus (see 2.3.3.1., 2.2.1.3. for protocol) exhibited intense uniform staining along their entire length, with >70% of spermatozoa agglutinated (Figure 22). Agglutinations were variable in size but in some cases comprised several hundred spermatozoa. Spermatozoa within agglutinations were generally more intensely stained, particularly in the flagellar region, which appeared primarily to be responsible for sperm-sperm agglutination. A relatively small percentage of fixed ($20.5 \pm 3.1\%$ of 600 non-agglutinated cells examined, from 3 experiments) and unfixed ($16.5 \pm 2.6\%$ of 600 non-agglutinated cells examined from 3 experiments) spermatozoa exhibited patchy staining along their entire length.

3.4.3.2. Activity of Spermatozoa In-Vitro Following Neuraminidase Treatment

Preliminary incubation of spermatozoa with neuraminidase at pH 6.0 and pH 7.4 (see 2.3.3.2. for protocol) indicated significantly better eosin exclusion (see 2.3.3.3.,

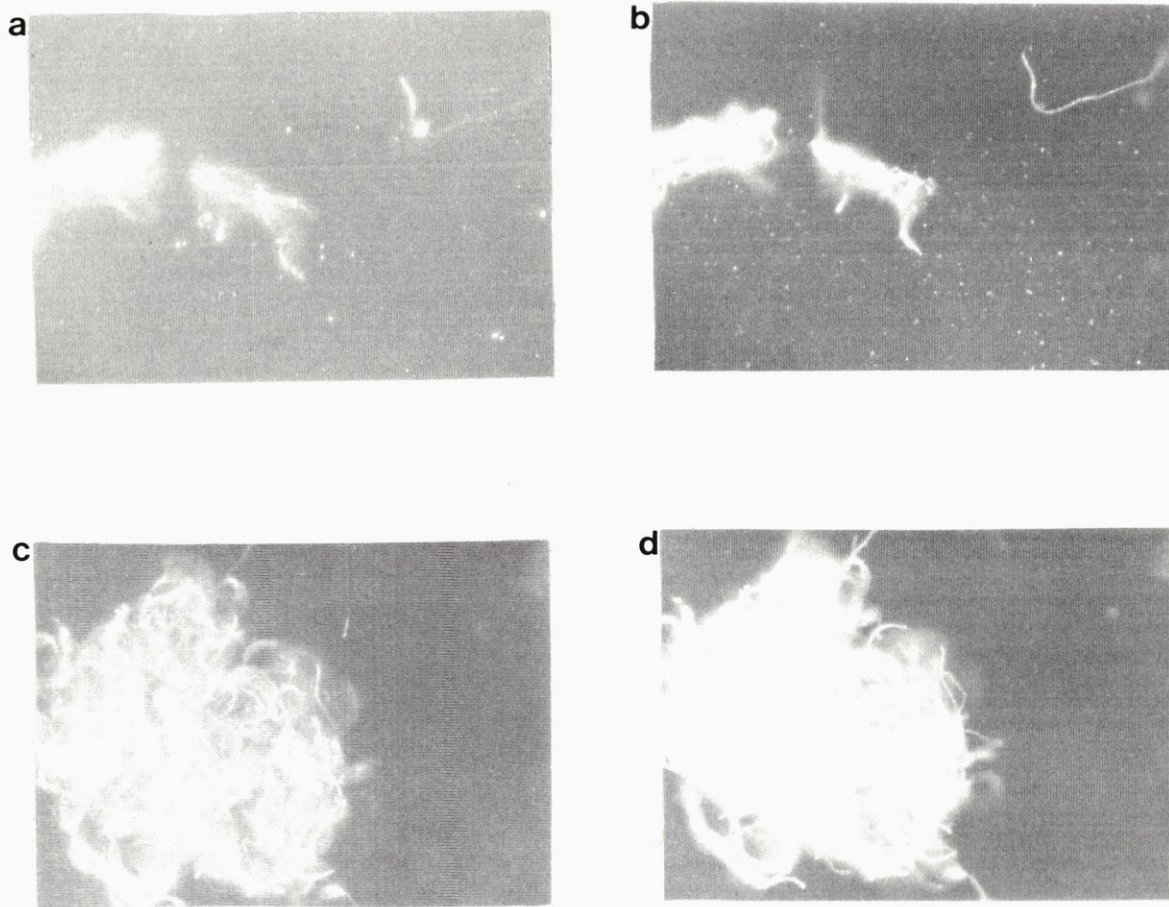


Figure 22. Photomicrographs of unfixed ejaculated spermatozoa stained with FITC conjugated lectin from Limulus polyphemus, taken using phase contrast(a,c) and epifluorescence(b,d) microscopy. X400.

2.3.1.2.3. for protocol) at pH 6.0 than at pH 7.4 ($P < 0.001$) (Table 7). Cellular ATP concentrations (see 2.3.3.3., 2.3.1.2.2. for protocol) were also higher in spermatozoa incubated at pH 6.0, although not significantly so ($P > 0.1$). Sperm motility (see 2.3.3.3., 2.3.1.2.1. for protocol) did not differ significantly with respect to pH ($P > 0.1$).

Eosin exclusion, cellular ATP concentration and sperm motility were not significantly different for control spermatozoa or spermatozoa treated with 40 I.U./ml neuraminidase, at either pH 6.0 or pH 7.4 ($P > 0.1$). However, cellular ATP concentrations were significantly reduced in samples assayed after 80 minutes incubation in all cases, compared with samples assayed after 20 minutes incubation subsequent to sperm treatment ($P < 0.001$) (Table 8).

| Spermatozoa | pH 6.0 | | | pH 7.4 | | |
|-----------------------|-------------------------------|-----------------------------|----------------------------------|-------------------------------|-----------------------------|----------------------------------|
| | % spermatozoa excluding eosin | ATP (nmol/ 10^9 sperm) | Motility (% $[\Delta OD]_m$) | % spermatozoa excluding eosin | ATP (nmol/ 10^9 sperm) | Motility (% $[\Delta OD]_m$) |
| control | 89.3 \pm 1.0 | 6.7 \pm 0.8 | 54.1 \pm 3.1 | 78.5 \pm 0.8 | 5.9 \pm 1.2 | 55.7 \pm 7.0 |
| neuraminidase treated | 86.6 \pm 0.8 | 6.8 \pm 0.9 | 47.6 \pm 3.6 | 76.5 \pm 0.8 | 6.1 \pm 0.9 | 53.6 \pm 7.3 |

Table 7. Effect of neuraminidase treatment at pH 6.0 and pH 7.4 on sperm function in-vitro. Parameters were measured during a 30 minute aerobic incubation at 40 °C, following treatment of spermatozoa with 40 I.U./ml neuraminidase. Values are mean \pm s.e.m. of data obtained from 3 experiments.

Given that spermatozoa incubated at pH 6.0 appeared to maintain their integrity in-vitro to a greater extent than those incubated at pH 7.4, and that neuraminidase exhibits optimal activity at acidic pH (see Froman & Engel, 1989), inseminations were subsequently carried out using spermatozoa treated with neuraminidase at pH 6.0.

| Spermatozoa | pH | 20 minute incubation | 80 minute incubation |
|-----------------------|-----|----------------------|----------------------|
| control | 6.0 | 6.7±0.8 | 1.4 |
| neuraminidase treated | 6.0 | 6.8±0.9 | 0.3 |
| control | 7.4 | 5.9±1.2 | 0.6 |
| neuraminidase treated | 7.4 | 6.1±0.9 | 0.7 |

Table 8. Effect of prolonged aerobic incubation at 40 °C of spermatozoa previously treated with 40 I.U./ml neuraminidase at pH 6.0 or pH 7.4, on cellular ATP concentration (nmol/10⁹ spermatozoa). Values are mean ± s.e.m. of data obtained from 3 experiments.

3.4.3.3. Activity of Spermatozoa In-Vivo Following Neuraminidase Treatment

Neuraminidase-treated spermatozoa were found in only 0.1% of uterovaginal junction SSTs (see 2.3.3.3., 2.3.1.2.4. for protocol) examined following intravaginal insemination (mean ± s.e.m. from 4 experiments, with an average of 111 SSTs examined per experiment) (Figure 23) compared with 43.1 ± 3.6% for control spermatozoa (of an

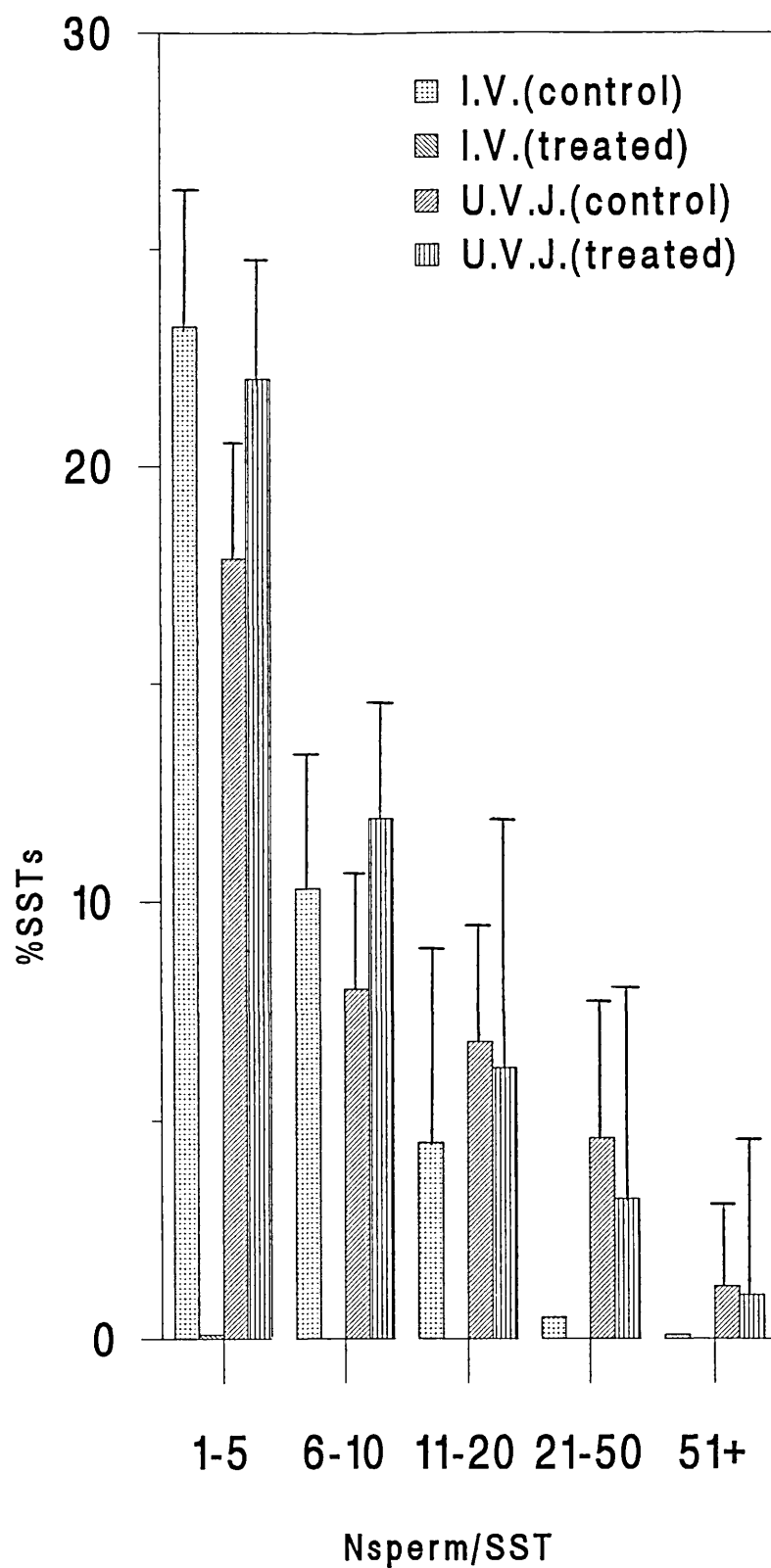


Figure 23. Access of spermatozoa to the Uterovaginal Junction SSTs following treatment with neuraminidase(40I.U./ml).Values are mean+/-s.e.m. from 3 experiments.

average of 121 SSTs examined per experiment) ($P < 0.001$). Furthermore, neuraminidase-treated spermatozoa were seldom observed on the uterovaginal junction mucosa. However, the percentages of SSTs containing neuraminidase-treated ($50.5 \pm 4.8\%$ of an average of 101 SSTs examined per experiment) and control ($37.9 \pm 5.4\%$ of an average of 103 SSTs examined per experiment) spermatozoa following their insemination into the uterovaginal junction lumen were not significantly different ($P > 0.1$).

3.4.4. Effect of Glycerol Treatment on Sperm Function
In-Vitro

3.4.4.1. Activity of Spermatozoa In-Vitro, Following
Treatment with Glycerol at Room Temperature and
at 5 °C

Cellular ATP concentration, sperm morphology and ability to exclude eosin (see 2.3.4.2., 2.3.1.2.2., 2.3.1.2.3. for protocols) did not differ significantly between spermatozoa treated with glycerol at room temperature (see 2.3.4.1. for protocol) and control spermatozoa ($P>0.1$) after 30 or 60 minutes of aerobic incubation at 40 °C, subsequent to glycerol removal. However, motility (see 2.3.4.2., 2.3.1.2.1. for protocol) after 10 minutes aerobic incubation was significantly reduced in all samples containing glycerol treated spermatozoa, in a glycerol concentration dependent manner ($P<0.05$) (Figures 24-27). Values for all parameters measured in glycerol-treated spermatozoa after 60 minutes aerobic incubation did not differ significantly from values obtained after 10 minutes or 30 minutes incubation ($P>0.1$) (Figures 28-31), but control sperm motility was significantly reduced ($P<0.01$).

Cellular ATP concentration, sperm morphology, ability to exclude eosin and motility did not differ significantly between spermatozoa treated with glycerol at 5 °C and control spermatozoa ($P>0.1$) after 30 or 60 minutes aerobic

incubation at 40 °C (Figures 24-31). For all parameters measured, values obtained at 60 minutes did not differ significantly from values obtained at 30 minutes ($P>0.1$).

After 30 and 60 minutes incubation, cellular ATP concentration, sperm morphology and eosin exclusion did not differ significantly between spermatozoa exposed to glycerol at 5 °C or at room temperature ($P>0.1$) (Figures 24-31). However, spermatozoa exposed to glycerol at 5 °C exhibited significantly greater motility after 1 and 60 minutes incubation than those exposed to glycerol at room temperature ($P<0.05$) with the exception of those treated with 0.5 and 2.0% glycerol, which showed no significant difference after 60 minutes aerobic incubation ($P>0.1$).

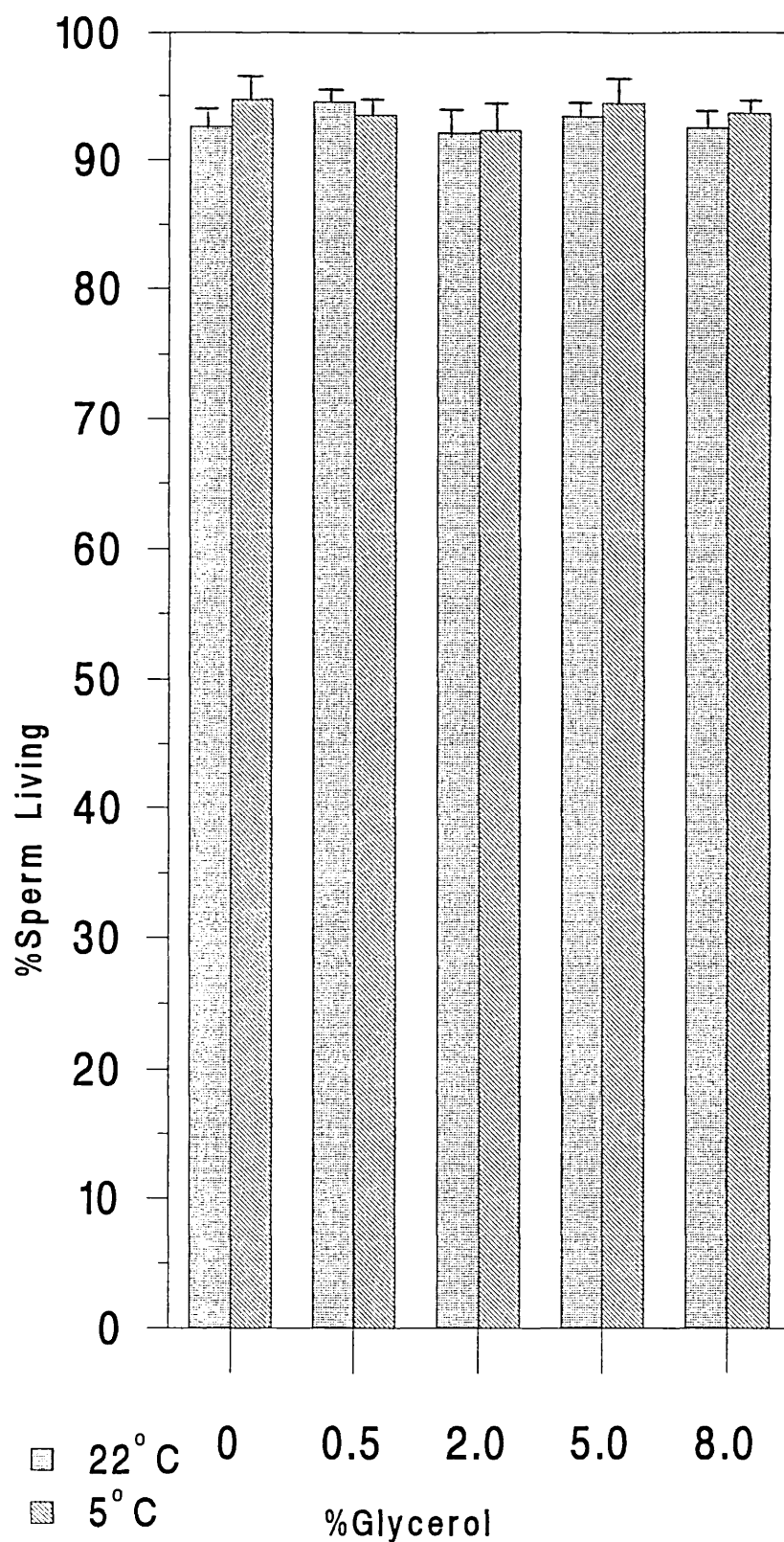


Figure 24. Effect of glycerol on sperm live/dead status at 22°C and 5°C. Values are mean \pm s.e.m. of data obtained during a 30 minute incubation at 40°C following glycerol removal, from 3 experiments.

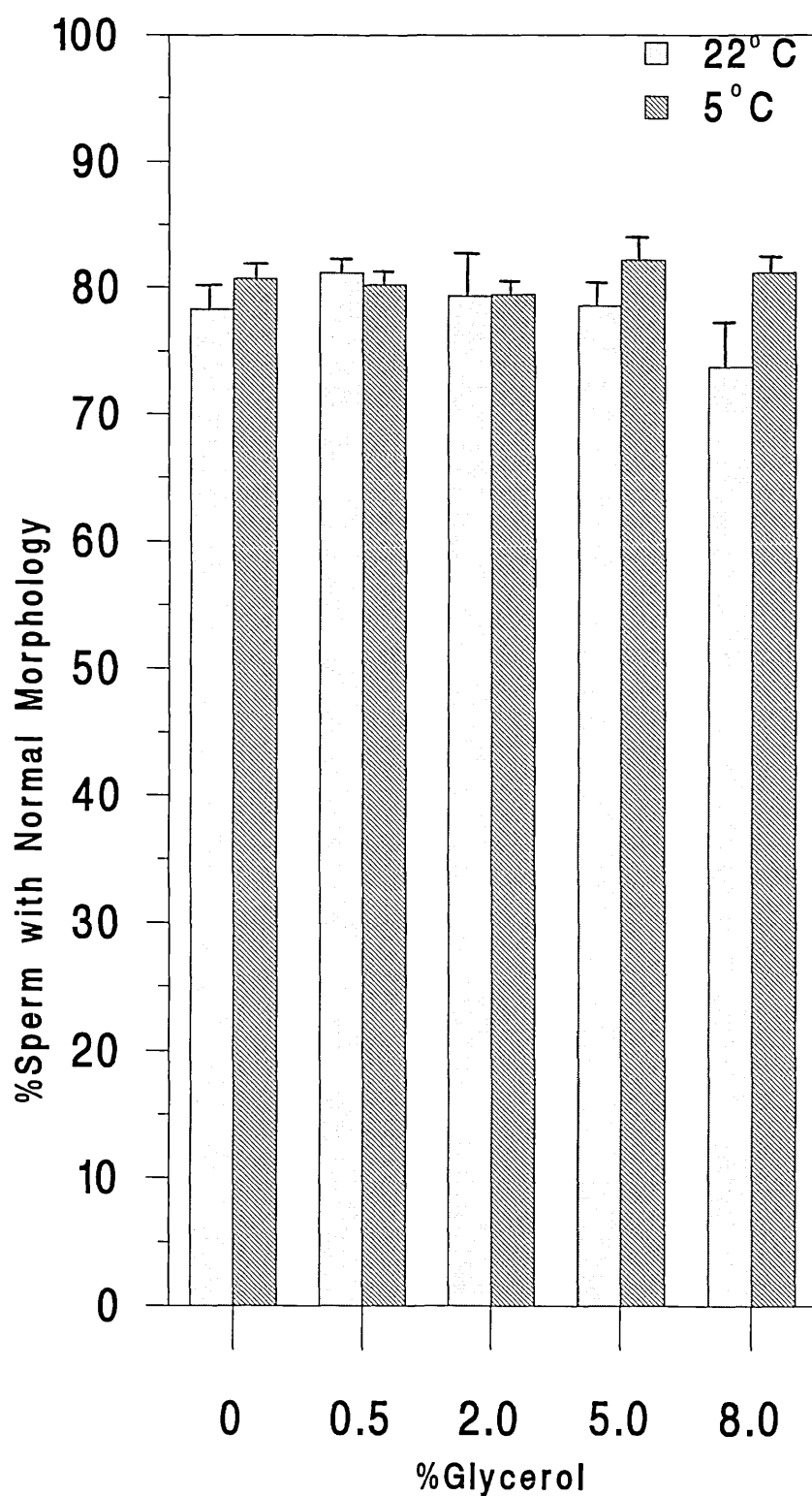


Figure 25. Effect of glycerol on sperm morphology in-vitro at 22° C and 5° C. Values are mean \pm s.e.m. of data obtained during a 30 minute incubation at 40° C following glycerol removal, from 3 experiments.

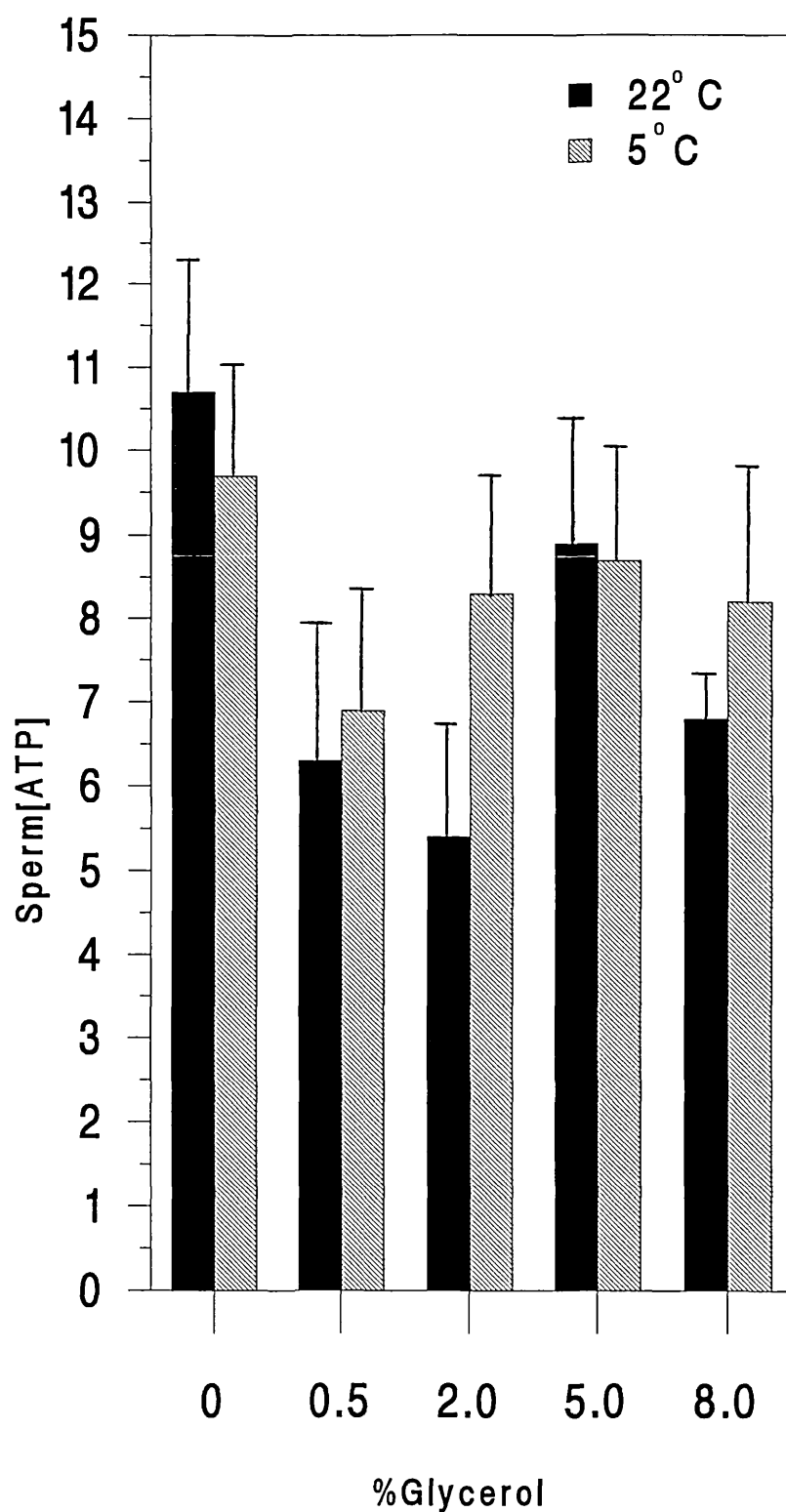


Figure 26. Effect of glycerol on sperm[ATP](nmol/10⁹sperm) in-vitro at 5° C and 22° C.Values are mean+/-s.e.m. of data obtained during a 30 minute incubation at 40° C following glycerol removal, from 3 experiments.

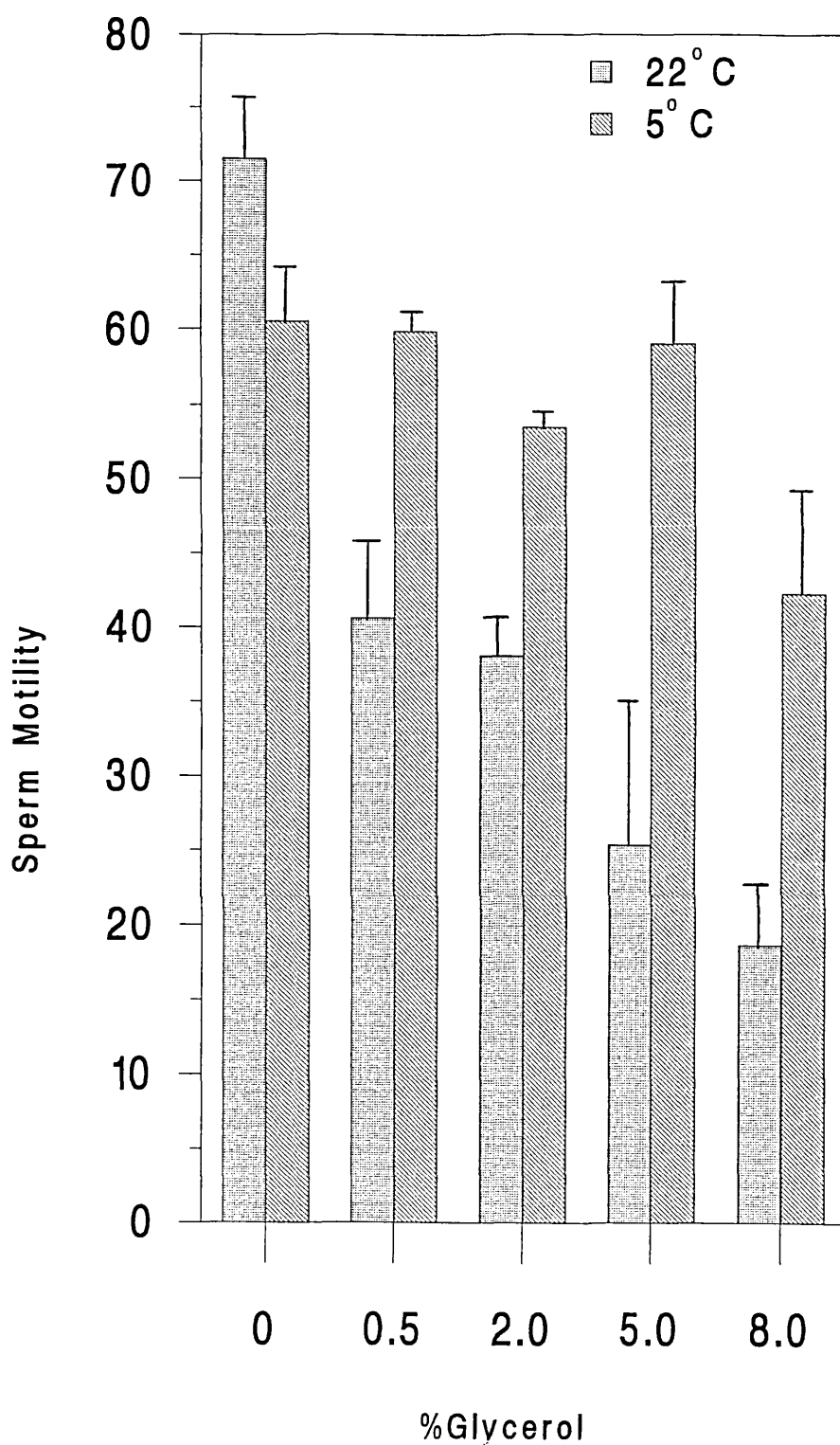


Figure 27. Effect of glycerol on sperm motility(% Δ OD/m) in-vitro at 22°C and 5°C. Values are mean \pm s.e.m. of data obtained after a 10 minute incubation at 40°C following glycerol removal, from 3 experiments.

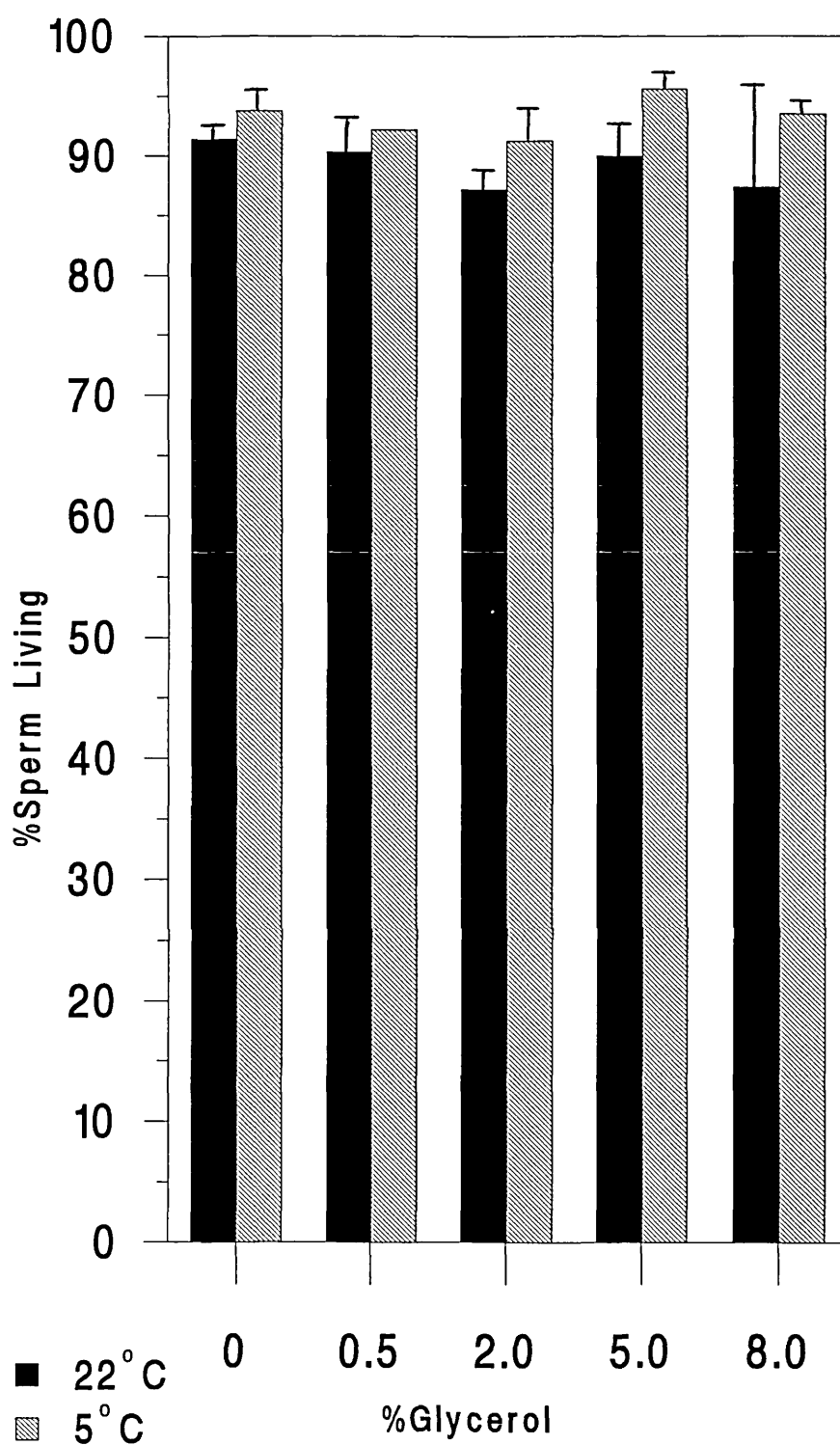


Figure 28. Effect of glycerol on sperm live/dead status in-vitro at 5°C and 22°C. Values are mean \pm s.e.m. of data obtained after a 60 minute incubation at 40°C following glycerol removal, from 3 experiments.

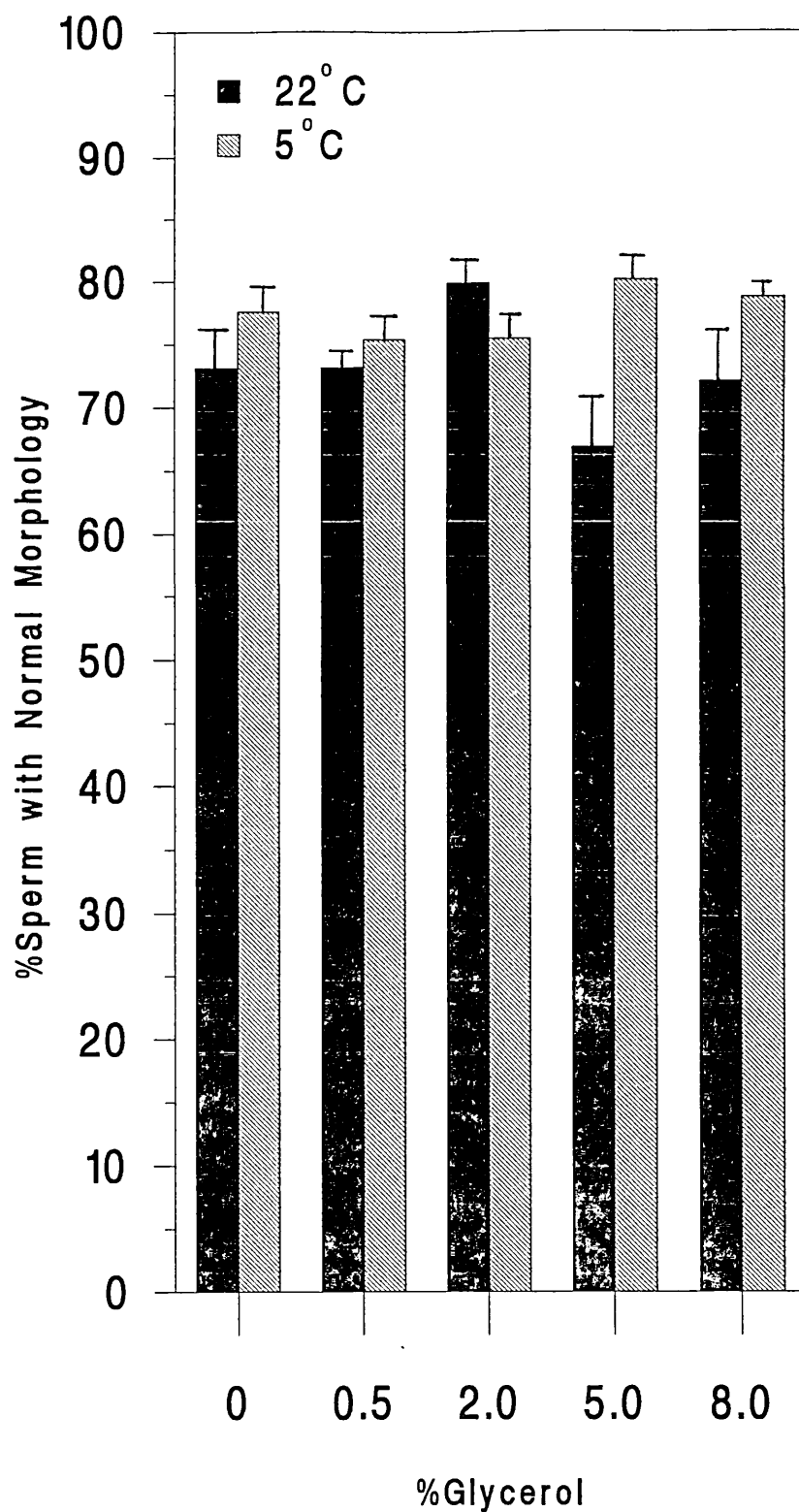


Figure 29. Effect of glycerol on sperm morphology in-vitro at 5°C and 22°C. Values are mean \pm s.e.m. of data obtained after a 60 minute incubation at 40°C following glycerol removal, from 3 experiments.

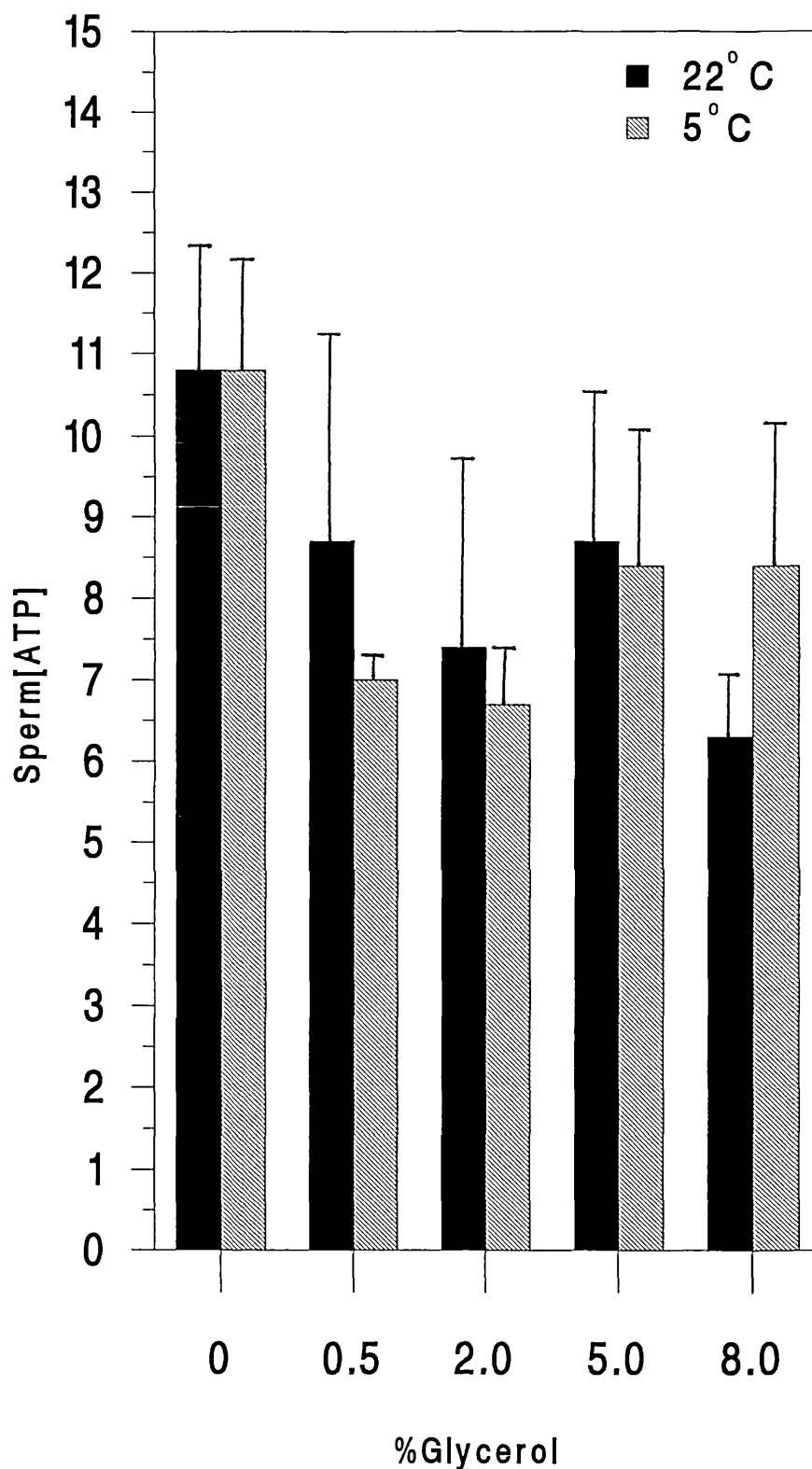


Figure 30. Effect of glycerol on sperm[ATP](nmol/10⁹ sperm) in-vitro at 5°C and 22°C. Values are mean \pm s.e.m. of data obtained after a 60 minute incubation at 40°C following glycerol removal, from 3 experiments.

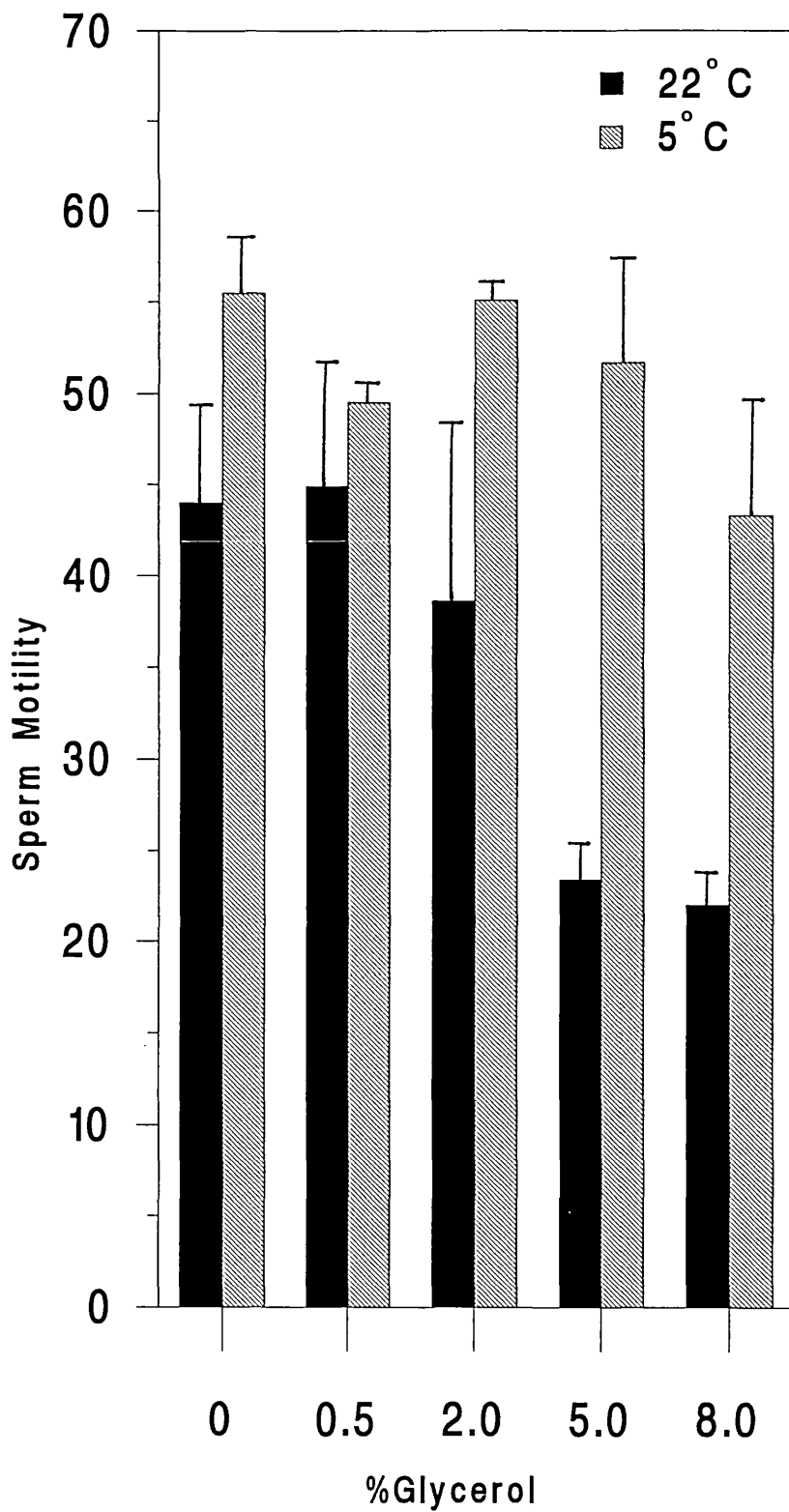


Figure 31. Effect of glycerol on sperm motility(% Δ OD)m in-vitro at 5° C and 22° C.Values are mean \pm -s.e.m. of data obtained after a 60 minute incubation at 40° C following glycerol removal, from 3 experiments.

3.4.4.2. Electrophoretic Profiles of Sperm Surface Washings Obtained from Glycerol Treatment of Spermatozoa

Clear differences were observed between the electrophoretic profiles of sperm surface washings from glycerol-treated and control spermatozoa (see 2.3.4.3., 2.2.2.2. for protocol) (Figure 32). Protein bands were observed at 46, 60, 63, 64 and 66 kd in control profiles obtained at room temperature. A number of extra bands were evident in the 5.0% glycerol treated profile including relatively strongly stained bands at 21, 25, 30, 53 and 68 kd, and more weakly stained bands at 40 and 43 kd. Bands at 21 and 25 kd were stained more intensely in the 8.0% glycerol treated profile and were accompanied by the appearance of a band at 22 kd. However, the band at 63 kd in the control profile was absent from both glycerol-treated profiles.

Protein bands observed on electrophoretic profiles of sperm surface washings obtained from control spermatozoa at 5 °C were generally of comparable staining intensity to those obtained at room temperature. However, sperm surface-associated proteins obtained from spermatozoa treated with 5.0 and 8.0% glycerol at 5 °C showed considerably weaker staining than their counterparts obtained at room temperature. Furthermore, considerably fewer bands appeared on profiles of sperm surface washings obtained from glycerol-treated spermatozoa, with extra

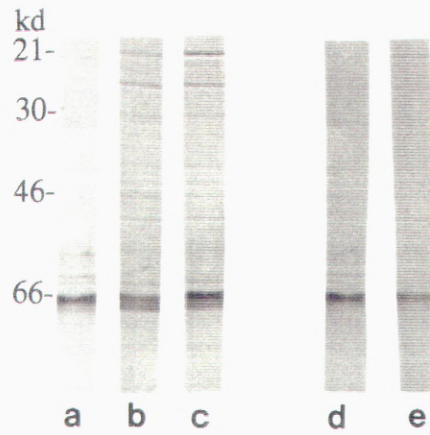


Figure 32. Silver stained electrophoretic profiles of sperm surface washings obtained from control spermatozoa washed with isotonic buffer(a), and spermatozoa exposed to 5.0%(b) and 8.0%(c) glycerol at room temperature(22°C). Lanes d and e are sperm surface washings obtained from control spermatozoa washed with isotonic buffer and spermatozoa exposed to 8.0% glycerol respectively, at 5°C.

bands at 21, 25 and 53 kd. Bands at 63 and 64 kd on the control profile were absent from the profiles of sperm surface washings obtained from glycerol-treated spermatozoa. Profiles of 5.0% (not shown) and 8.0% glycerol-treated sperm surface washings exhibited identical banding and very similar staining intensities.

Electrophoretic profiles of sperm surface washings obtained from treatment of spermatozoa with 0.5 and 2.0% glycerol at room temperature and 5 °C (not shown), showed similar staining patterns and intensities to control profiles.

3. 5. Oviducal Sperm Selection

3. 5. 1. Entry of Homologous and Heterologous Spermatozoa to the Uterovaginal Junction SSTs In-Vitro

Quail uterovaginal junction SSTs were used because chicken uterovaginal junction SST cells contain large numbers of vesicles which increase SST opacity, hindering examination of tubule contents. Although avian spermatozoa could be positively identified within chicken SSTs, mammalian spermatozoa could not. However, quail uterovaginal junction SSTs are generally devoid of such vesicles and allowed easy identification of mammalian spermatozoa contained within their lumen.

Despite differences in sperm length, motility characteristics and morphology, spermatozoa from all homologous and heterologous samples used were found in quail uterovaginal junction SSTs after co-incubations in-vitro (see 2.6.1.1., 2.6.1.2. for protocol) (Table 9, Figure 33). The percentage of SSTs containing spermatozoa ranged from 3% for rat epididymal spermatozoa to 64% for human ejaculated spermatozoa, with mammalian spermatozoa generally performing as well as their avian counterparts. Abnormal forms of chicken spermatozoa were observed in 44.9% of 118 SSTs examined (see 2.6.1.5., 2.3.1.1., 2.6.1.1., 2.6.1.2. for protocol). Both avian and mammalian spermatozoa observed at the tip of SSTs adopted

the storage orientation described by Van Krey et al. (1981), lying parallel to each other with lateral head contact. As with avian spermatozoa, most mammalian spermatozoa observed within SSTs rapidly became quiescent, and no spermatozoa were observed leaving SSTs.

Prior to incubation, ejaculated spermatozoa generally demonstrated greater uniformity of motility within semen samples than did testicular, epididymal (mammalian) or ductus deferens spermatozoa, as assessed by light microscopy. Rat epididymal spermatozoa very quickly became immotile in the medium used.

Vigorous ciliary activity was observed on uterovaginal junction mucosal tissue throughout the duration of shaken and unshaken incubations, indicating good tissue viability.

| Semen used | Type of incubation | No. spermatozoa | No. SSTs examined | % SSTs containing spermatozoa |
|-------------------------|--------------------|-----------------------|-------------------|-------------------------------|
| chicken testicular | non-shaken | 20 X 10 ⁶ | 110 | 28 |
| chicken ductus deferens | non-shaken | 50 X 10 ⁶ | 70 | 54 |
| chicken ejaculated | non-shaken | 10 X 10 ⁶ | 71 | 45 |
| quail ductus deferens | non-shaken | 120 X 10 ⁶ | 87 | 34 |
| mouse epididymal | non-shaken | 20 X 10 ⁶ | 140 | 33 |
| rat epididymal | non-shaken | 20 X 10 ⁶ | 75 | 3 |
| bovine ejaculated | non-shaken | 10 X 10 ⁶ | 90 | 63 |
| human ejaculated | non-shaken | 10 X 10 ⁶ | 86 | 64 |
| chicken ejaculated | shaken | 10 X 10 ⁶ | 55 | 40 |
| turkey ejaculated | shaken | 10 X 10 ⁶ | 57 | 35 |
| human ejaculated | shaken | 10 X 10 ⁶ | 56 | 54 |

Table 9. Filling of quail uterovaginal junction SSTs in-vitro. Values are data from single experiments.

3.5.2. Cross-reactivity of Antiserum Raised Against Chicken Ejaculated Seminal Plasma Proteins, with Bovine and Human Spermatozoa

The antiserum raised in rabbit against chicken ejaculated seminal plasma proteins did not visibly cross-react with

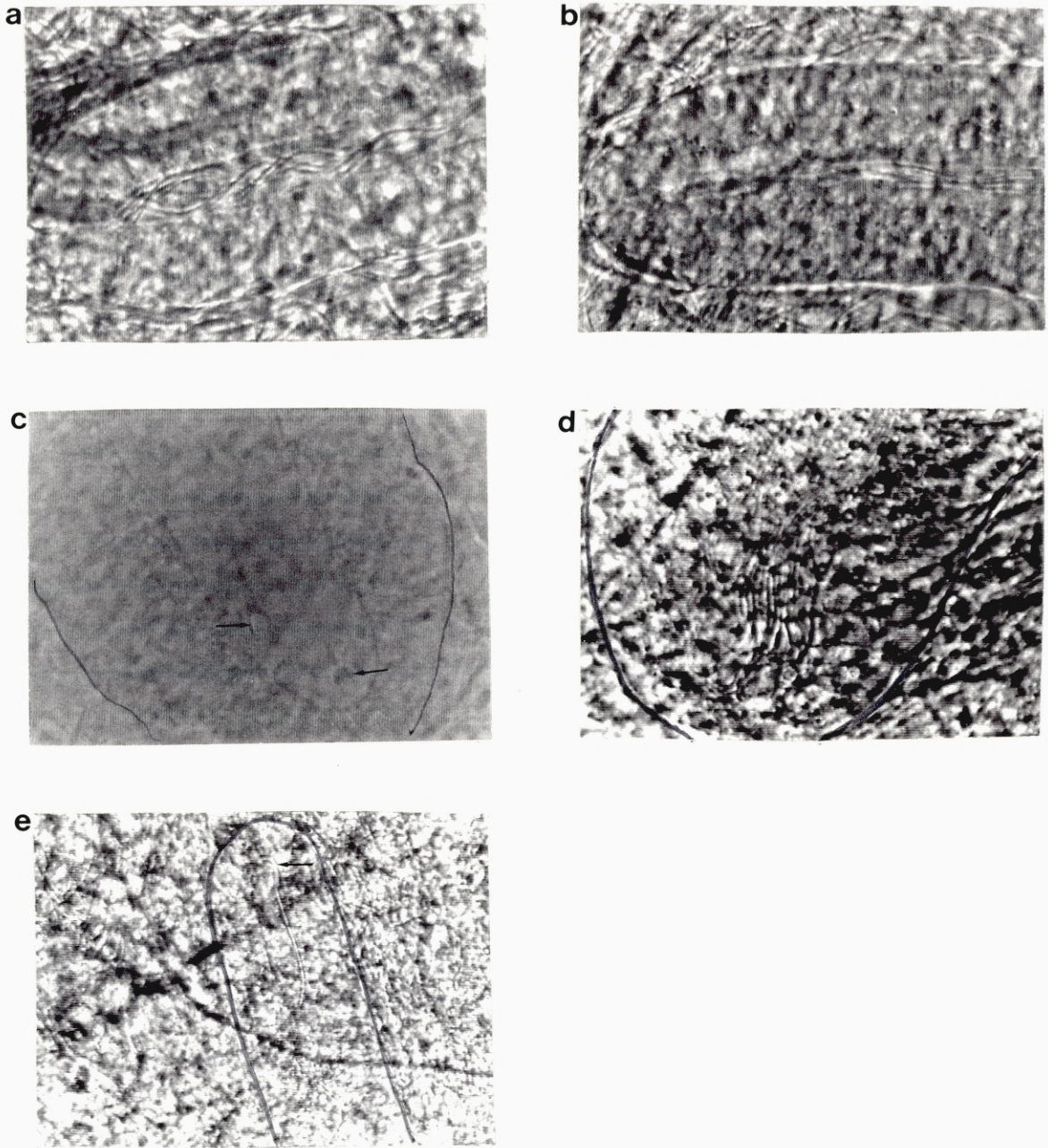


Figure 33. Photomicrographs of quail uterovaginal junction SSTs containing quail(a), chicken(b), human(c), bovine(d) and rat(e) spermatozoa following in-vitro incubation, taken using phase contrast microscopy.X1000.

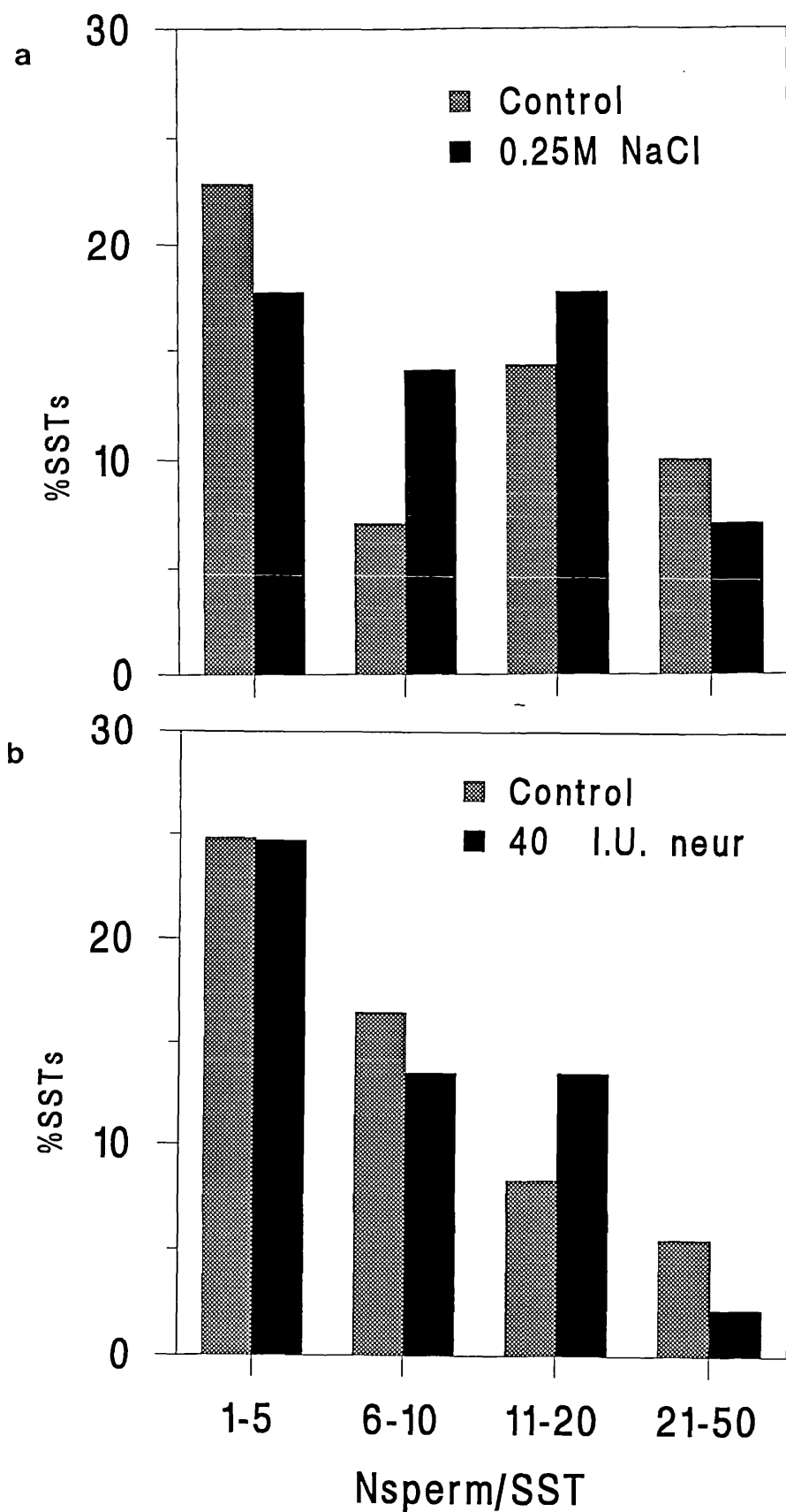


Figure 34. Access of hypertonic(0.25M NaCl)(a), and neuraminidase(40I.U./ml)(b) treated spermatozoa to the uterovaginal junction SSTs in-vitro.Values are data from single experiments.

surface antigens on the mammalian spermatozoa tested (see 2.6.1.4., 2.2.1.2. for protocol) indicating a lack of antigenic similarity between avian and mammalian sperm surface associated proteins (Figure 35).

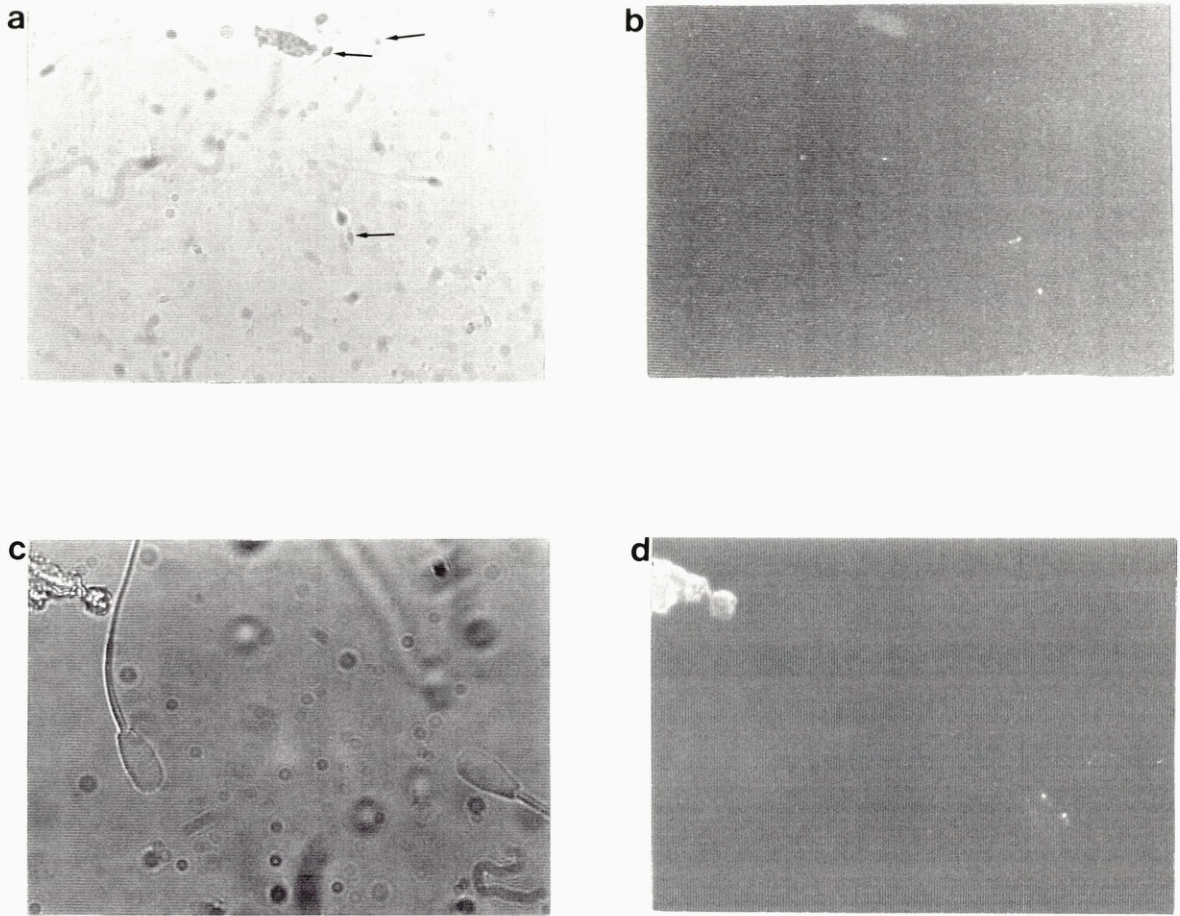


Figure 35. Photomicrographs of human(a,b) and bovine(c,d) spermatozoa stained with antiserum raised against ejaculated chicken seminal plasma proteins, and FITC conjugated goat antirabbit IgG. Photographs were taken using phase contrast(a,c) and epifluorescence microscopy. X400 (chicken), X1000 (bovine).

3. 5. 3. Identification of the Site of Oviducal Sperm Selection

3. 5. 3. 1. Cross-reactivity of Antiserum Raised Against Chicken Seminal Plasma Proteins with Turkey Spermatozoa

Both chicken and turkey spermatozoa were stained after exposure to antiserum raised against ejaculated chicken seminal plasma proteins, and subsequently FITC-conjugated goat antirabbit IgG (see 2. 6. 2. 2. 1., 2. 2. 1. 2. for protocol). Chicken spermatozoa exhibited intense uniform staining along their entire length. Turkey spermatozoa were less intensely or uniformly stained and exhibited mainly head, midpiece and anterior flagellar staining (Figure 36).

Following exhaustive absorption of the antiserum with turkey spermatozoa, chicken spermatozoa subsequently exposed to the antiserum continued to stain along their entire length (Figure 37) (see 2. 6. 2. 2. 2., 2. 2. 1. 2. for protocol). Staining appeared considerably more variable than with untreated antiserum, ranging from light speckling along the flagellum with more intense staining of the head and midpiece regions, to intense uniform staining along the entire length of the spermatozoa.

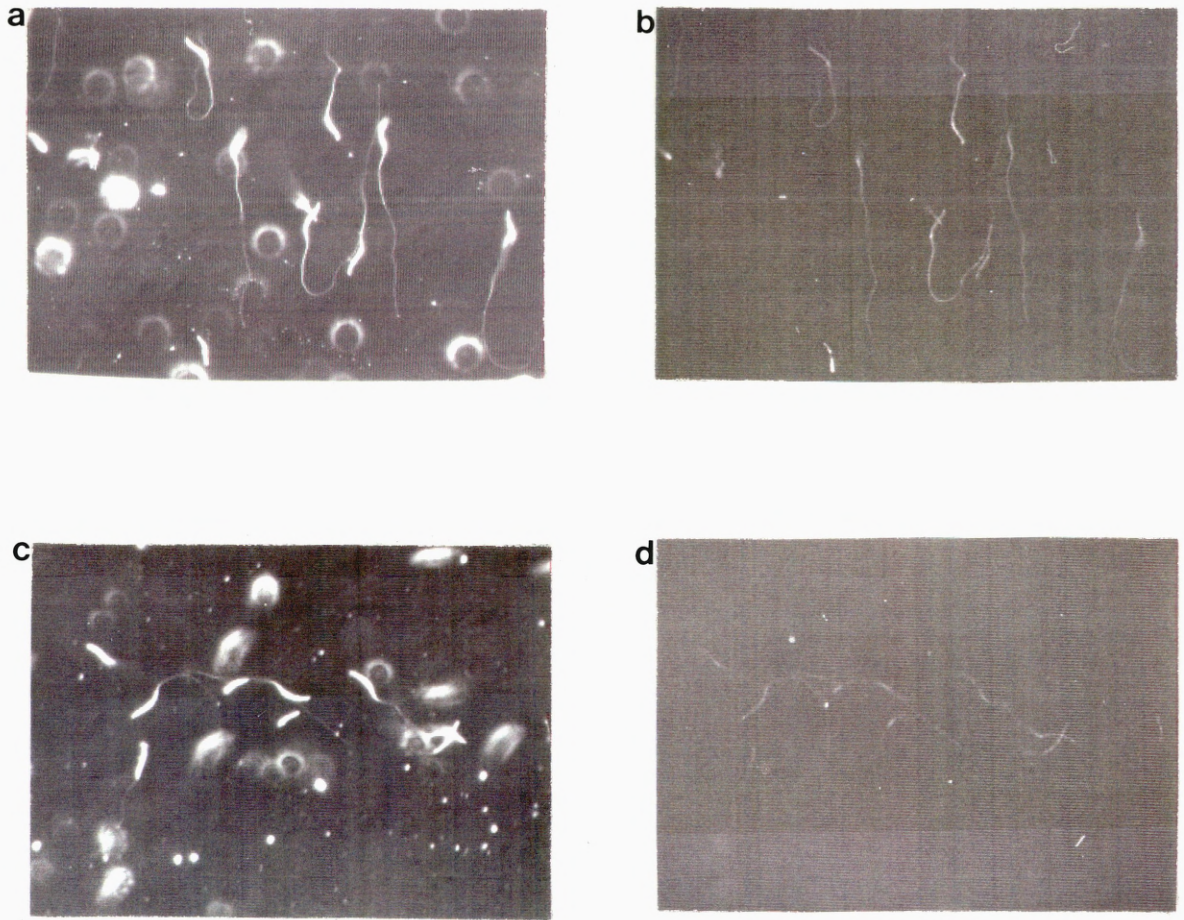


Figure 36. Photomicrographs of chicken(a,b) and turkey(c,d) spermatozoa stained with rabbit antiserum raised against ejaculated chicken seminal plasma proteins, and FITC conjugated goat antirabbit IgG. Photographs were taken using phase contrast(a,c) and epifluorescence(b,d) microscopy.X400.

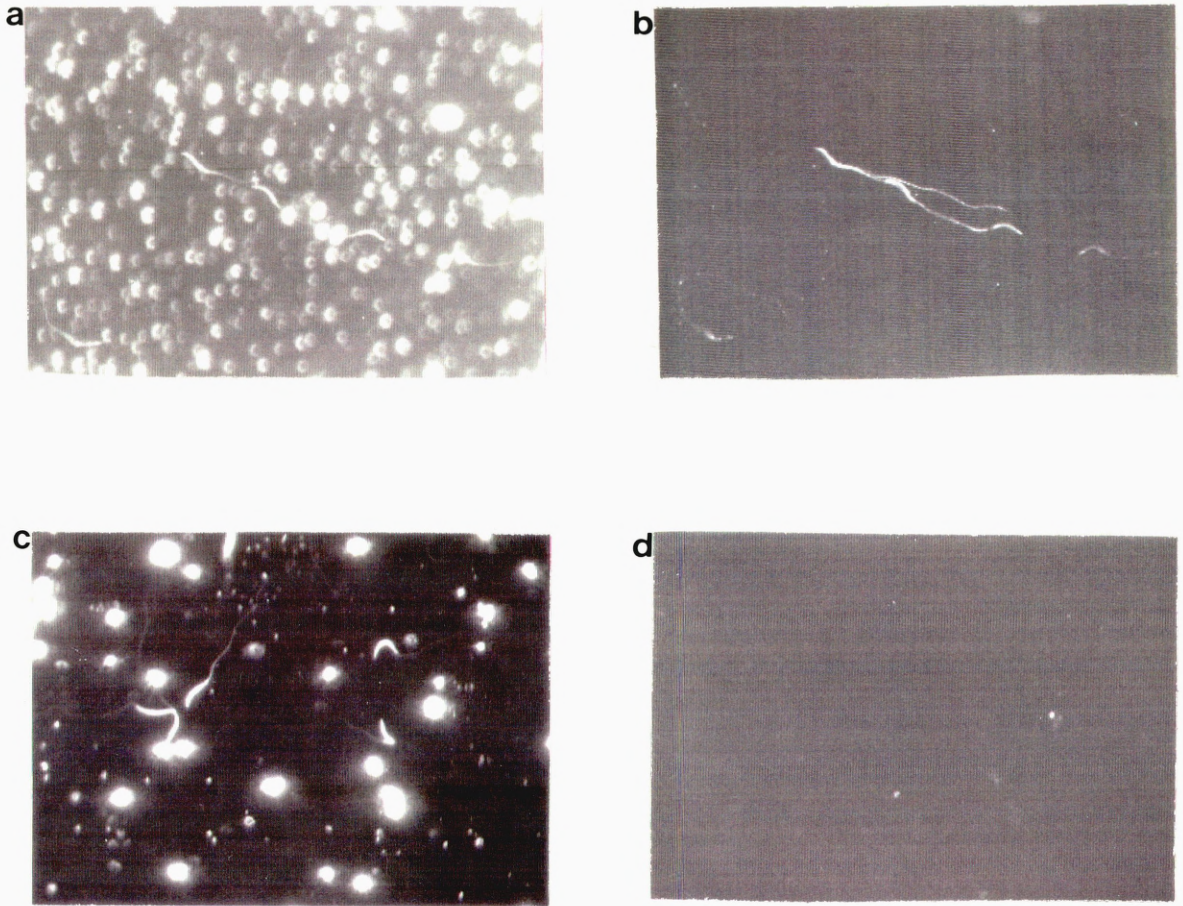


Figure 37. Photomicrographs of chicken(a,b) and turkey(c,d) spermatozoa stained with rabbit antiserum raised against ejaculated chicken seminal plasma proteins, absorbed with turkey spermatozoa, and FITC conjugated goat antirabbit IgG. Photographs were taken using phase contrast(a,c) and epifluorescence(b,d) microscopy.X400.

3.5.3.2. Timed Entry of Chicken and Turkey Spermatozoa,
and Entry of Hypertonic Buffer-Treated and
Neuraminidase-Treated Chicken Spermatozoa to
Chicken Uterovaginal Junction SSTs In-Vitro

During a timed incubation, turkey spermatozoa entered and populated chicken uterovaginal junction SSTs in-vitro (see 2.6.2.1., 2.6.1.1., 2.6.1.2., 2.3.1.2.4. for protocol) at a rate at least equivalent to, if not greater than the rate at which SSTs were populated by homologous chicken spermatozoa (Figures 38, 39). Furthermore, the distribution of spermatozoa throughout the SSTs appeared similar for both species, with continued filling of SSTs throughout the incubation.

Both chicken and turkey spermatozoa exhibited vigorous forward motility throughout the duration of the incubation, as assessed by light microscopy.

Chicken spermatozoa previously treated with buffer containing either 0.25M NaCl (see 2.3.1.1. for protocol) or 40 I.U./ml neuraminidase (see 2.3.3.2. for protocol) were found in 57.7% (85 SSTs examined) and 53.9% (98 SSTs examined) respectively, of chicken uterovaginal junction SSTs examined (see 2.6.1.3., 2.3.1.2.4. for protocol), compared with 54.3% (70 SSTs examined) and 55.0% (80 SSTs examined) for their respective controls. Distribution of

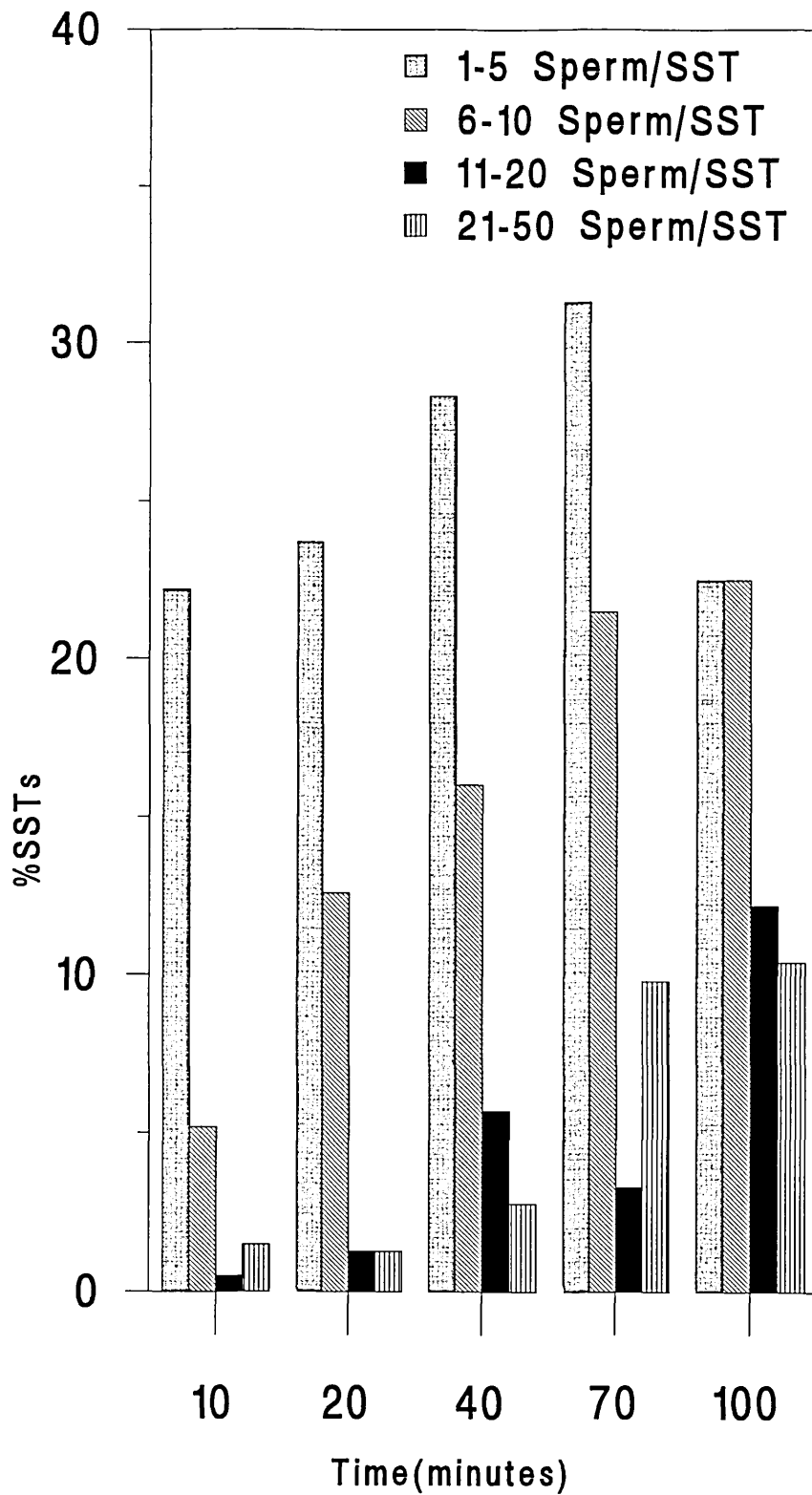


Figure 38. Time course of filling of chicken Uterovaginal Junction SSTs with turkey spermatozoa *in-vitro*. Values are means from 2 experiments.

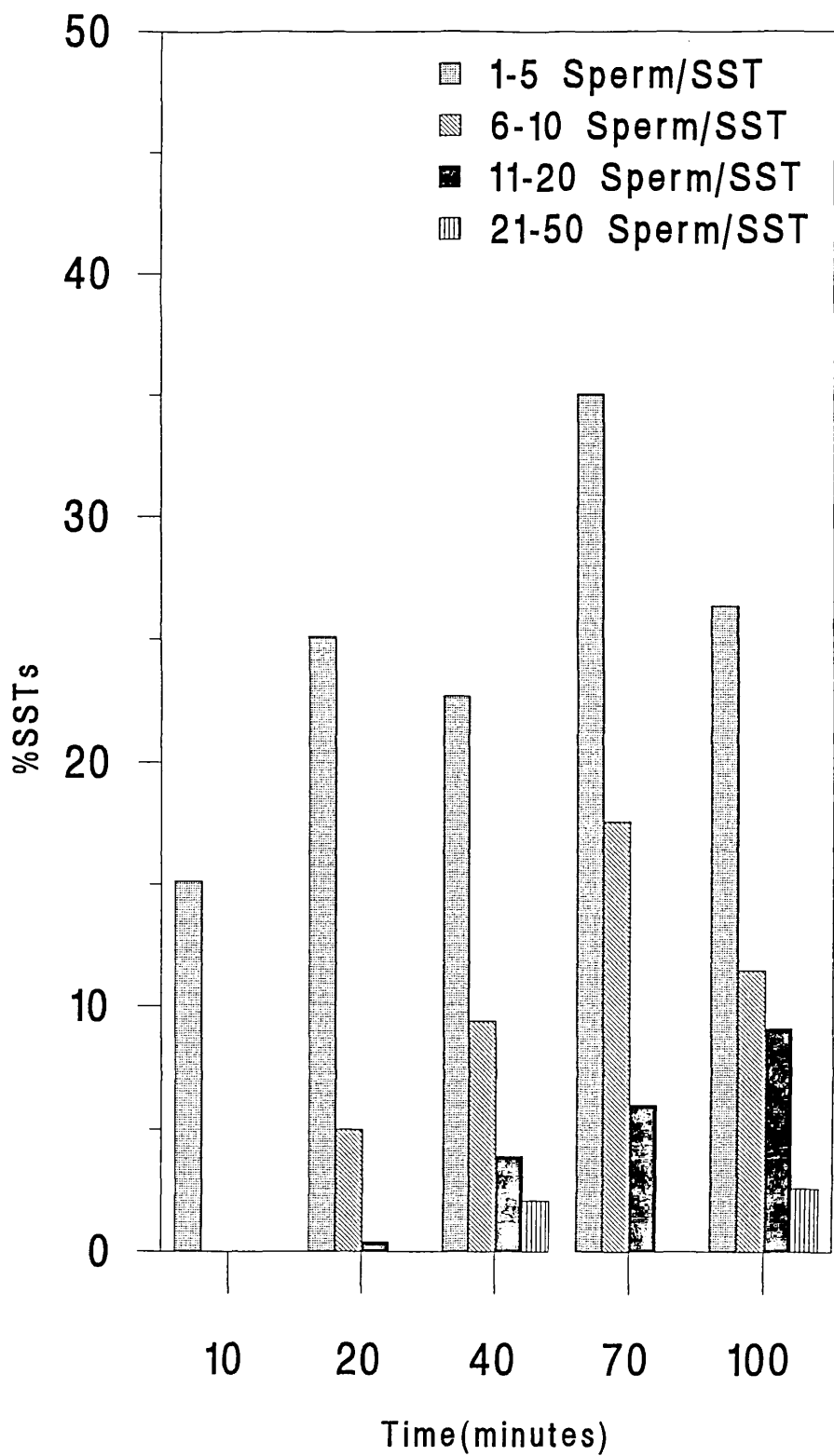


Figure 39. Time course of filling of chicken Uterovaginal Junction SSTs with chicken spermatozoa *in-vitro*. Values are means from 2 experiments.

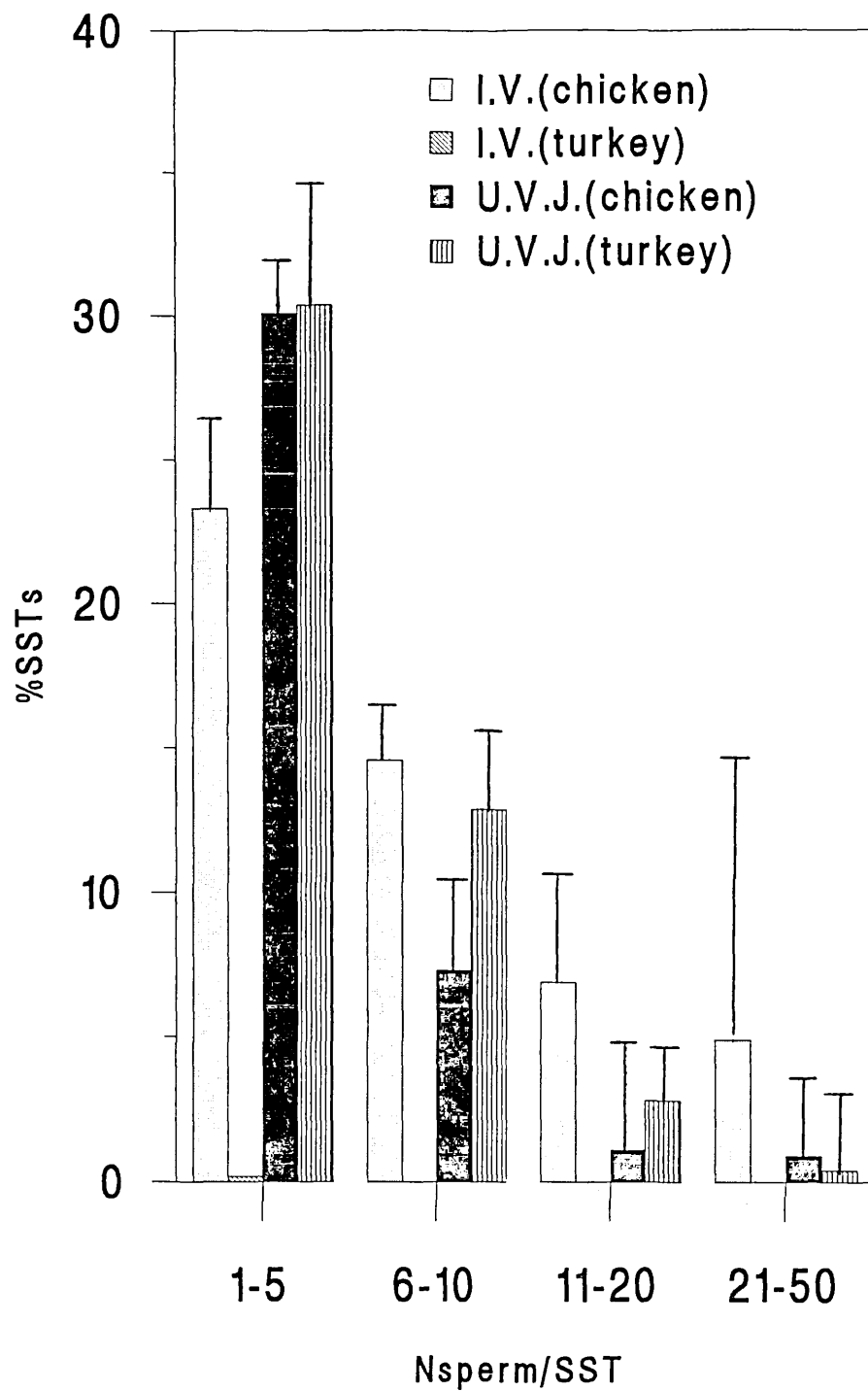


Figure 40. Access of chicken and turkey spermatozoa to the chicken U.V.J.SSTs in-vivo, following I.V. or U.V.J. insemination. Values are mean \pm s.e.m. of data from 3(chicken sperm) or 6(turkey sperm) hens. An average of 105 SSTs were assessed from each hen.

spermatozoa throughout SSTs appeared variable for treated spermatozoa and their respective controls (Figure 34).

Ciliary activity of uterovaginal junction tissue remained vigorous throughout all incubations, indicating good tissue viability.

3.5.3.3. Entry of Homologous and Heterologous Spermatozoa to Chicken and Quail Uterovaginal Junction SSTs In-Vivo

Following intravaginal insemination of chicken or turkey spermatozoa into chicken hens, only $0.2 \pm 0.1\%$ of uterovaginal junction SSTs examined (see 2.6.2.3., 2.3.1.2.4. for protocol) contained turkey spermatozoa, compared with $50.1 \pm 6.3\%$ for chicken spermatozoa ($P < 0.001$) (Figure 40). However, following insemination into the lumen of the uterovaginal junction, $48.5 \pm 3.6\%$ of SSTs contained turkey spermatozoa, compared with $40.9 \pm 1.7\%$ for chicken spermatozoa ($P > 0.1$). The distributions of chicken and turkey spermatozoa throughout the SSTs were similar after uterovaginal junction insemination.

Human spermatozoa were observed in 4.8% of quail uterovaginal junction SSTs (83 SSTs examined) from one of the 2 hens examined (see 2.6.1.6., 2.6.1.2. for protocol). Spermatozoa observed in SSTs, although intact, exhibited

rigid flagellar conformation and appeared to be floating free within the tubule lumen, suggesting that such spermatozoa may have been dead. No spermatozoa were observed in SSTs from the other hen. Small numbers of spermatozoa were observed in the outer perivitelline layer of eggs from 4 of the other 5 hens inseminated (see 2.6.1.6., 2.3.1.2.5. for protocol), and eggs from a single hen were observed to contain spermatozoa for 4 consecutive days following insemination (Table 10). Sperm nuclei stained intensely with DAPI, and spermatozoa appeared intact by phase contrast microscopy 5 days following insemination (Figure 41).

| Hen No. | Day 2 | Day 3 | Day 4 | Day 5 |
|---------|-------|-------|-------|-------|
| 1 | 2.2 | 1.3 | 0.5 | 0.2 |
| 2 | 0.5 | 0.0 | 0.0 | 0.0 |
| 3 | 0.8 | 0.0 | 0.0 | 0.0 |
| 4 | 0.0 | 0.0 | 0.0 | 0.0 |
| 5 | 0.5 | 0.0 | 0.0 | 0.0 |

Table 10. Access of intravaginally inseminated human spermatozoa to the quail egg in-vivo. Values represent the mean number of spermatozoa per 5.5 mm² of outer perivitelline layer, from 6 assessments per egg.

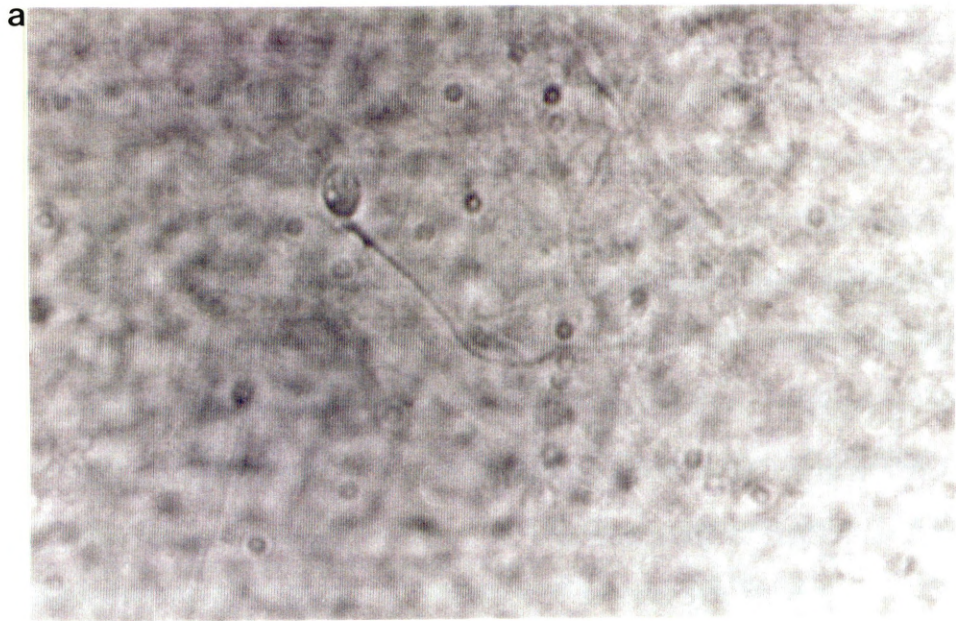


Figure 41. Photomicrographs of a human spermatozoon trapped in the outer perivitelline layer of a laid quail egg following intravaginal insemination, stained with DAPI. Photographs were taken using phase contrast(a) and epifluorescence(b) microscopy.X1000.

3. 5. 4. Potential Physiological Basis of Oviducal Sperm Selection

3. 5. 4. 1. Chicken Immunoglobulin binding In-Vivo and Live/Dead Status of Homologous and Heterologous Spermatozoa Recovered by Vaginal Lavage Following Intravaginal Insemination

A high percentage ($63.5 \pm 1.9\%$ of 3770 spermatozoa examined) of the $3.0 \pm 0.6 \times 10^6$ spermatozoa (mean \pm s.e.m. from 18 washings) recovered from the chicken vagina by lavage 20 minutes following insemination had chicken immunoglobulin bound to their surface (see 2.6.3.1. for protocol), a slightly lower percentage ($59.4 \pm 5.4\%$ of 715 spermatozoa examined) staining dead (see 2.6.3.1., 2.3.1.2.3. for protocol). These percentages increased with time to $96.7 \pm 6.0\%$ (of 334 spermatozoa examined) after 8 hours for immunoglobulin binding and $99.1 \pm 2.6\%$ (of 152 spermatozoa examined) after 18 hours for cells staining dead (Figure 42). In contrast, only $1.2 \pm 0.1\%$ of non-inseminated control spermatozoa (4500 spermatozoa examined) stained positively for bound immunoglobulin, with $4.0 \pm 0.7\%$ (of 900 spermatozoa examined) staining dead after 20 minutes incubation at 40 °C.

Binding of chicken immunoglobulin was variable with regard to intensity and pattern. Staining of head and midpiece regions was either uniformly speckled or patchy, whereas

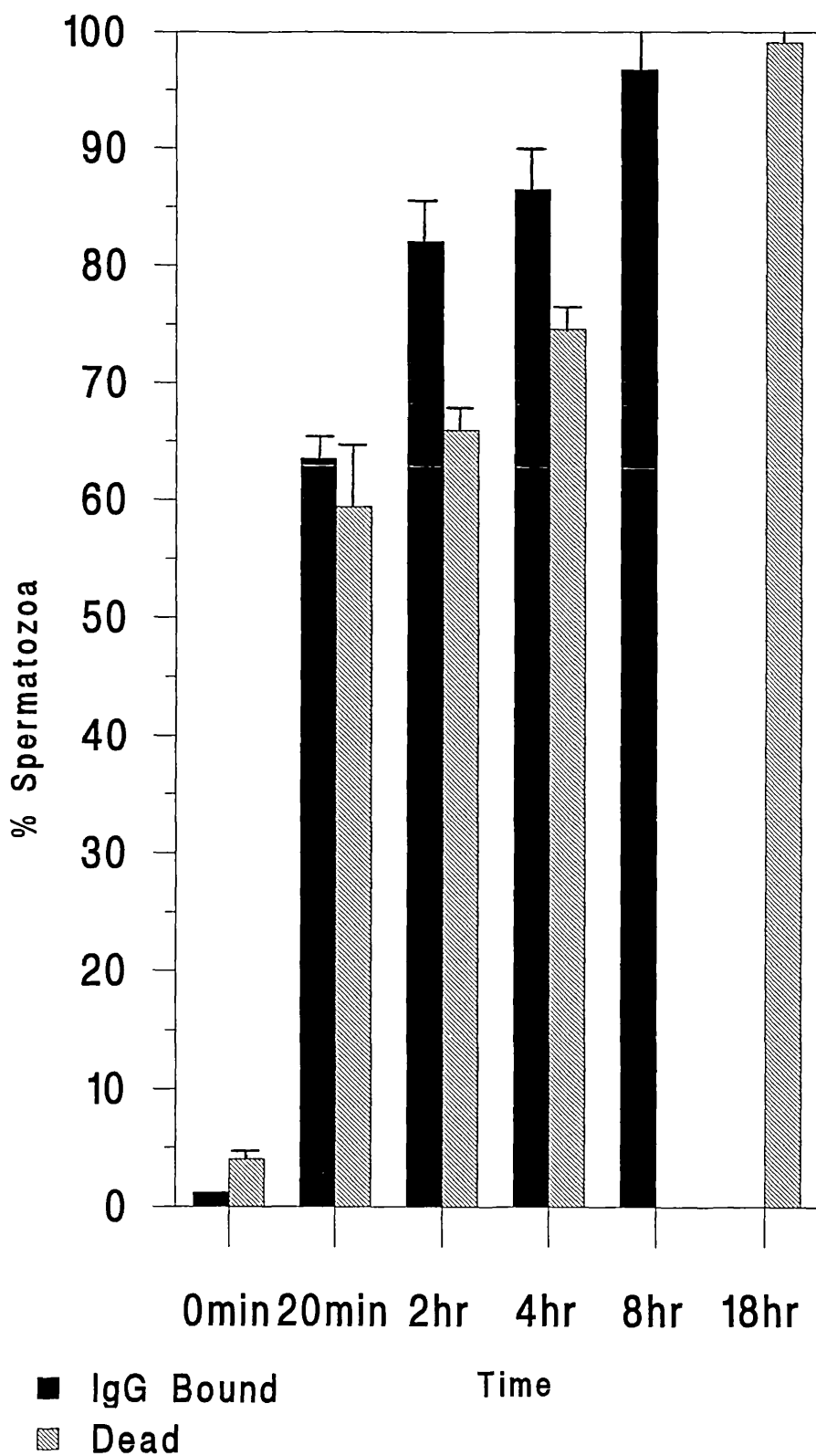


Figure 42. Chicken IgG binding and live/dead status of chicken spermatozoa recovered from the chicken vagina by lavage at timed intervals following I.V. insemination. Values are mean \pm s.e.m. from an average of 9 hens per time period.

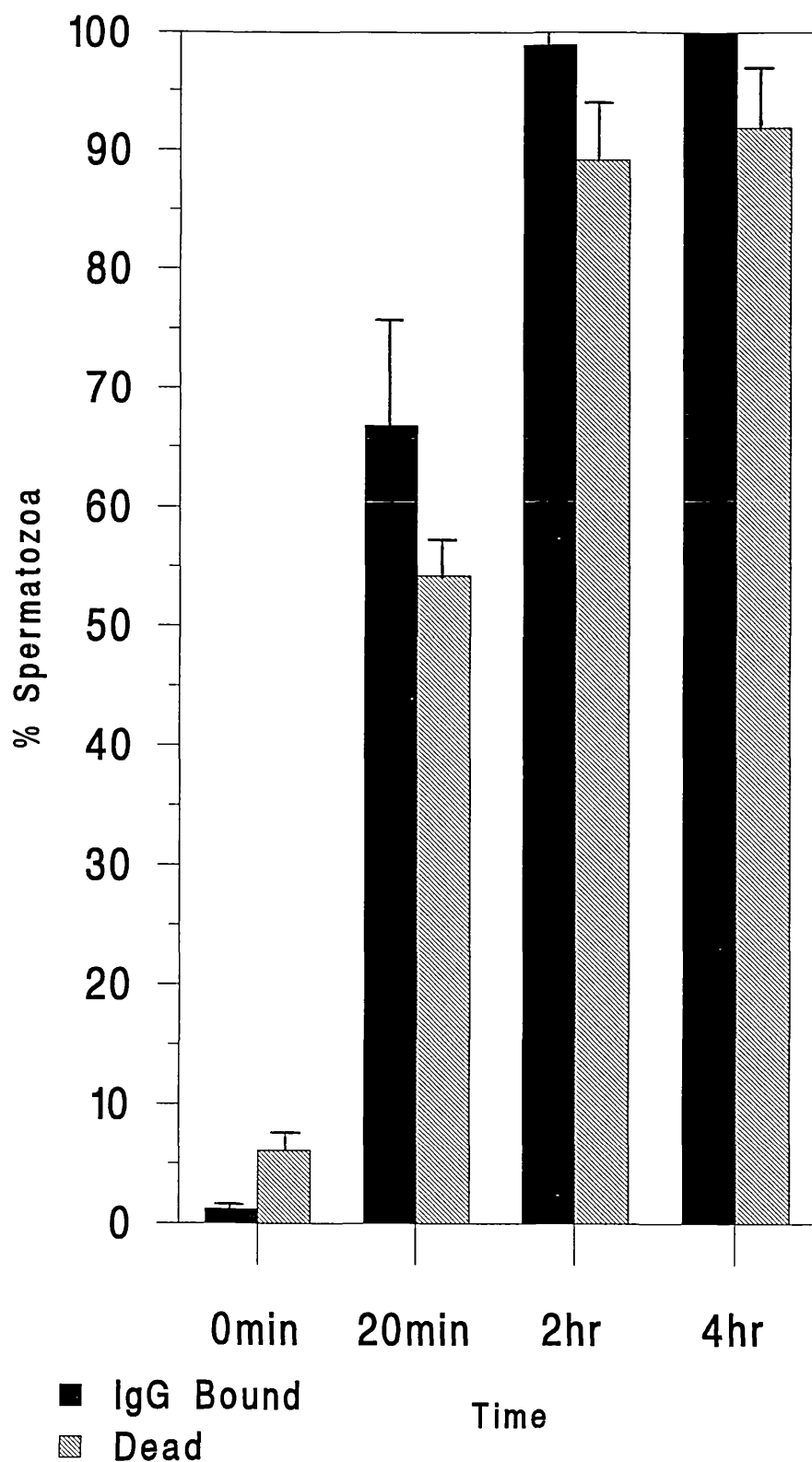


Figure 43. Chicken IgG binding and live/dead status of turkey spermatozoa recovered from the chicken vagina by lavage at timed intervals following I.V. insemination. Values are mean \pm s.e.m. from an average of 6 hens per time period.

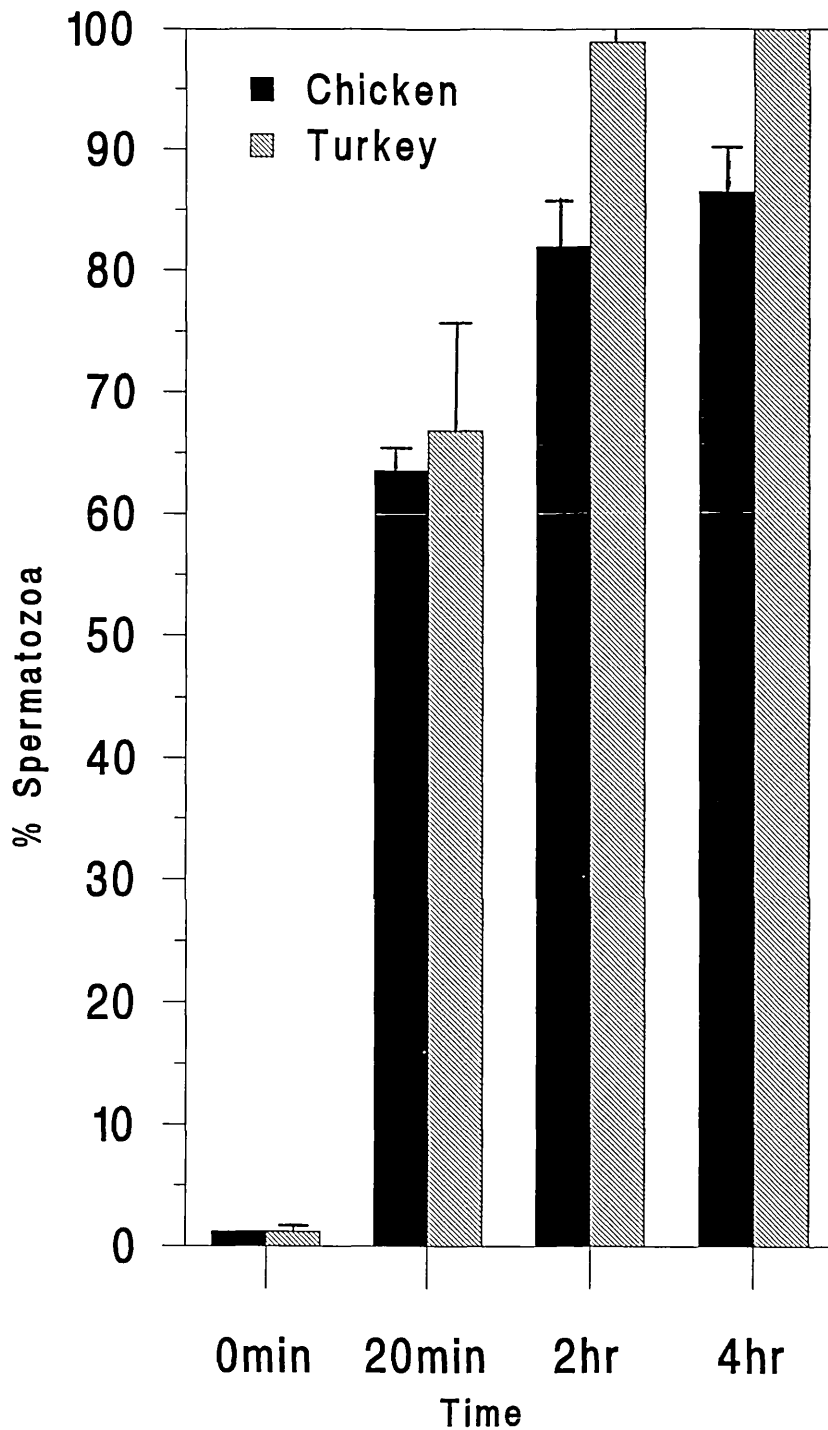


Figure 44. Chicken IgG binding to chicken and turkey spermatozoa recovered from the chicken vagina by lavage at timed intervals following I.V. insemination. Values are mean \pm s.e.m. from an average of 9(chicken sperm) or 6(turkey sperm) hens per time period

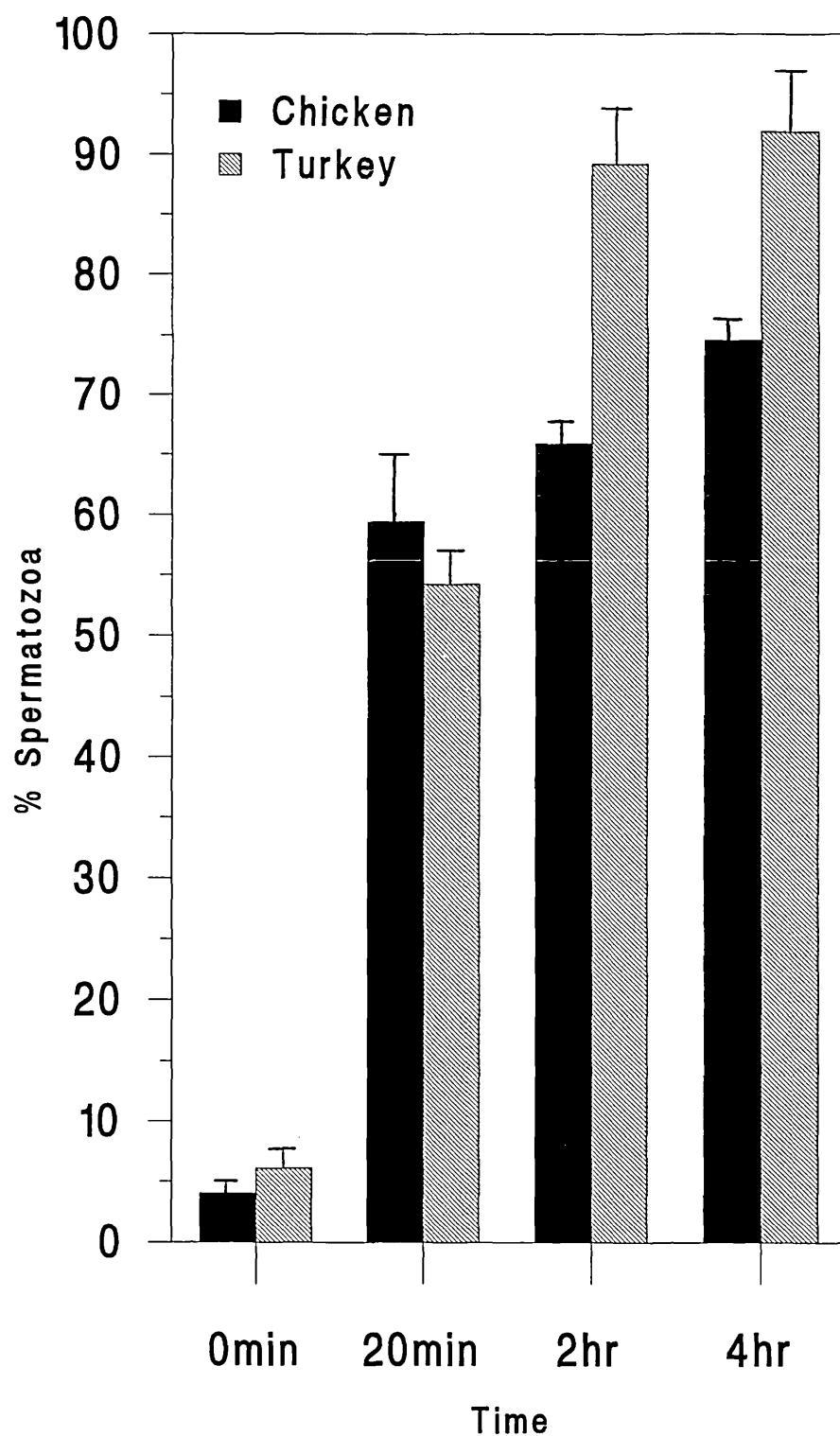


Figure 45. Live/dead status of chicken and turkey spermatozoa recovered from the chicken vagina by lavage at timed intervals following I.V. insemination. Values are mean \pm s.e.m. from an average of 9(chicken sperm) or 6(turkey sperm) hens per time period.

staining of the flagellar region was generally uniform, more intense, and observed along clearly demarcated areas (Figure 47).

Of spermatozoa recovered by vaginal lavage 20 minutes following intravaginal insemination, $40.5 \pm 1.8\%$ of those stained exhibited a combination of head and flagellar staining (Table 11). Flagellar staining within this group generally involved the anterior to mid-flagellar region, with some posterior flagellar staining. Smaller groups of spermatozoa exhibited head only or flagellar only staining, staining in the latter group being mainly restricted to the posterior flagellar region.

Intravaginal insemination of turkey spermatozoa into chicken hens produced similar results (Figure 43) but the percentages of lavaged spermatozoa with bound chicken immunoglobulin and staining dead increased to maxima over a shorter time period (Figures 44, 45). Of spermatozoa recovered by lavage 20 minutes following intravaginal insemination, 66.8 ± 8.7 (521 spermatozoa examined) had chicken immunoglobulin bound to their surface, with $54.2 \pm 2.7\%$ (810 spermatozoa examined) staining dead. These percentages increased with time to 100% (197 spermatozoa examined) after 4 hours for bound chicken immunoglobulin and $91.9 \pm 5.1\%$ (568 spermatozoa examined) for spermatozoa staining dead (Figure 43).

Only $1.2 \pm 0.3\%$ (1007 spermatozoa examined) of non-inseminated control spermatozoa stained positively for bound immunoglobulin, with $6.1 \pm 1.3\%$ (900 spermatozoa examined) staining dead.

Patterns of chicken immunoglobulin binding to turkey spermatozoa differed from those observed on chicken spermatozoa (Table 11, Figure 47). The percentage of spermatozoa recovered from the chicken vagina 20 minutes following insemination, with immunoglobulin bound to the head and flagellar regions, was similar to that observed for chicken spermatozoa (Table 11). However, flagellar staining mainly involved the anterior to mid-flagellar region. Furthermore, the percentage of spermatozoa demonstrating head-only staining was significantly lower, and the percentage of spermatozoa exhibiting flagellar-only staining significantly higher than for chicken spermatozoa ($P < 0.01$).

| Spermatozoa | head + flagellum | head only | flagellum only |
|-------------|------------------|----------------|----------------|
| chicken | 40.5 ± 1.8 | 24.6 ± 2.6 | 30.2 ± 1.8 |
| turkey | 38.5 ± 4.4 | 6.2 ± 5.1 | 52.9 ± 4.1 |

Table 11. Pattern of intravaginal binding of chicken immunoglobulin to chicken and turkey spermatozoa *in-vivo*. Values are mean \pm s.e.m. percentages of spermatozoa exhibiting regional immunoglobulin binding, from the examination of 3600 chicken spermatozoa from 18 hens and 521 turkey spermatozoa from 4 hens.

The percentages of spermatozoa recovered 20 minutes following insemination which had immunoglobulin bound to their surface or stained dead did not differ significantly between the 2 species ($P>0.1$). However, values for both parameters differed significantly between chicken and turkey spermatozoa recovered from the vagina 2 hours and 4 hours following insemination ($P<0.05$).

Assessments of immunoglobulin binding and live/dead status were carried out on free spermatozoa in washings. However, spermatozoa were often observed in agglutinations of various size or suspended in large numbers in vaginal mucus (Figure 46). All of such spermatozoa stained dead and most had immunoglobulin bound to their surface.

Sperm motility in vaginal washings varied among hens, but in general, $>80\%$ of spermatozoa recovered 20 minutes after insemination were immotile, with a small percentage ($<10\%$) exhibiting vigorous forward motility.

The percentage of spermatozoa recovered by vaginal lavage 20 minutes following insemination, which had immunoglobulin bound to their surface, did not differ significantly ($P>0.1$) between virgin hens ($58.5 \pm 2.3\%$ of 2800 spermatozoa examined, from 14 hens) and hens which had been inseminated on a number of occasions over preceding months ($60.8 \pm 2.2\%$ of 1300 spermatozoa examined, from 9 hens) (see 2.6.3.3., 2.6.3.1. for protocol). Similarly, the percentages of spermatozoa

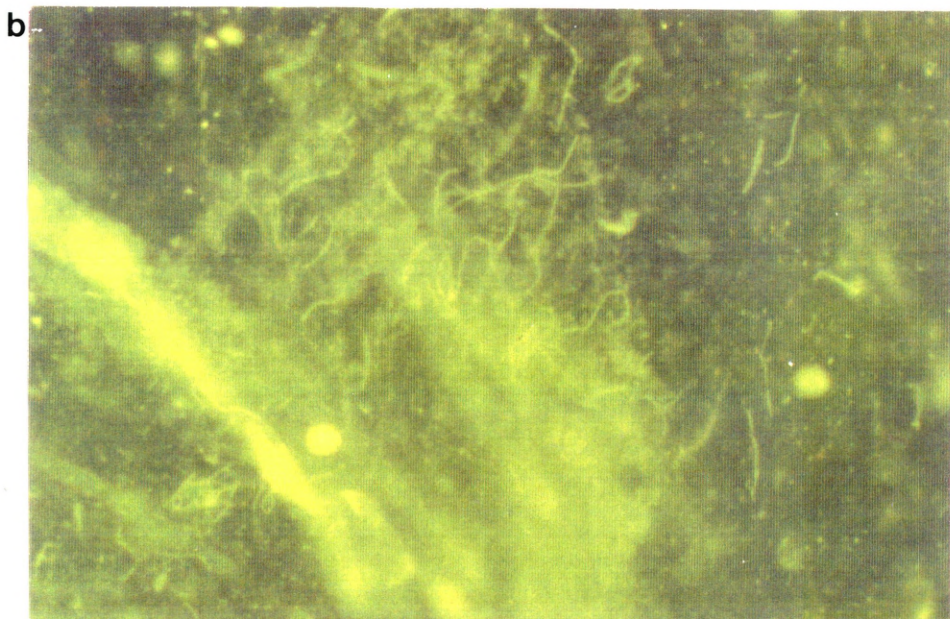
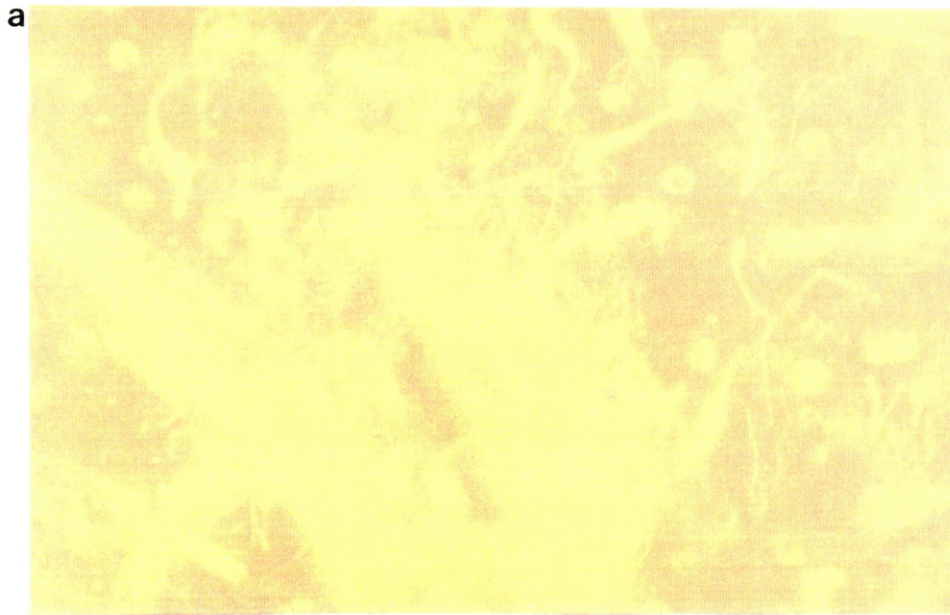


Figure 46. Photomicrographs of chicken spermatozoa suspended in vaginal mucus following recovery by vaginal lavage 20 minutes after intravaginal insemination, stained with FITC conjugated rabbit antichickn IgG. Photographs were taken using phase contrast(a) and epifluorescence(b) microscopy.X400.

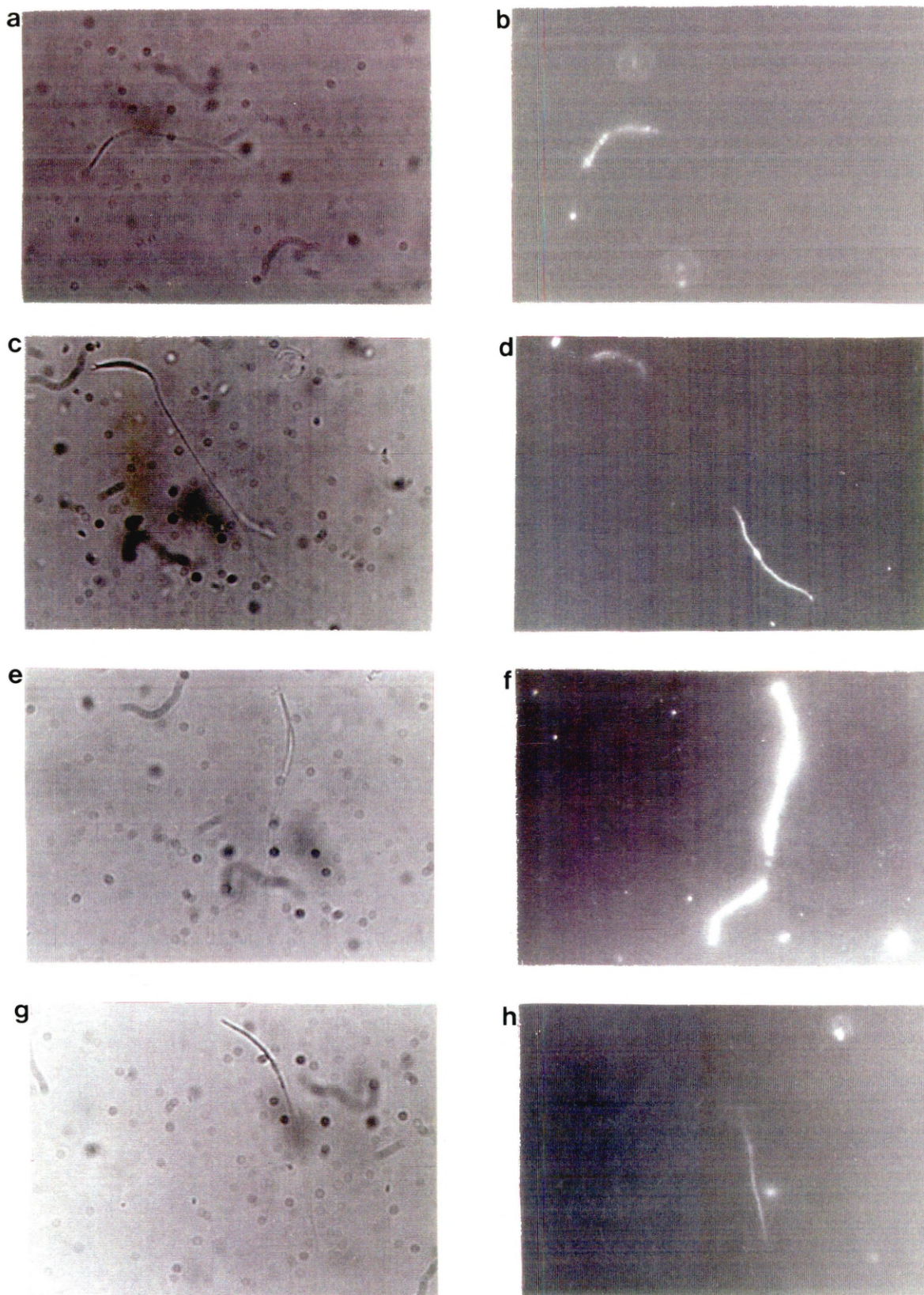


Figure 47. Photomicrographs of chicken(a,b,c,d) and turkey(e,f,g,h) spermatozoa recovered from the chicken vagina by lavage 20 minutes following intravaginal insemination, and stained with rabbit antichickan IgG. Photographs were taken using phase contrast(a,c,e,g) and epifluorescence(b,d,f,h) microscopy.X1000.

staining dead ($54.8 \pm 1.4\%$ of 335 spermatozoa examined, from 15 hens, for previously inseminated birds and $54.1 \pm 1.8\%$ of 622 spermatozoa examined, from 15 hens, for virgin birds) (see 2.6.3.3., 2.3.1.2.3. for protocol) did not differ significantly ($P > 0.1$).

Of human spermatozoa recovered from the chicken vagina 10 minutes after insemination, 85.6% (125 spermatozoa examined) stained positively for surface bound chicken immunoglobulin (Figure 48) (see 2.6.3.4., 2.6.3.1. for protocol), compared with 3.3% (400 spermatozoa examined) for non-inseminated controls. The pattern of staining on recovered spermatozoa was variable, as was staining intensity. All 400 control gerbil spermatozoa examined exhibited very faint uniform staining of the head and midpiece regions, whereas spermatozoa recovered from the vagina exhibited intense staining along their entire length (88 spermatozoa examined).

Leishmans stained vaginal smears obtained from 2 non-inseminated control hens (see 2.6.3.6. for protocol) showed large numbers of cilia torn from the vaginal mucosal cells, and some large epithelial cells. No other cell types were observed. Smears from 4 inseminated hens were similar, but generally contained spermatozoa in large numbers, which appeared intact. No macrophages were observed, although a small number of small rounded cells with large regular nuclei may have been lymphocytes.

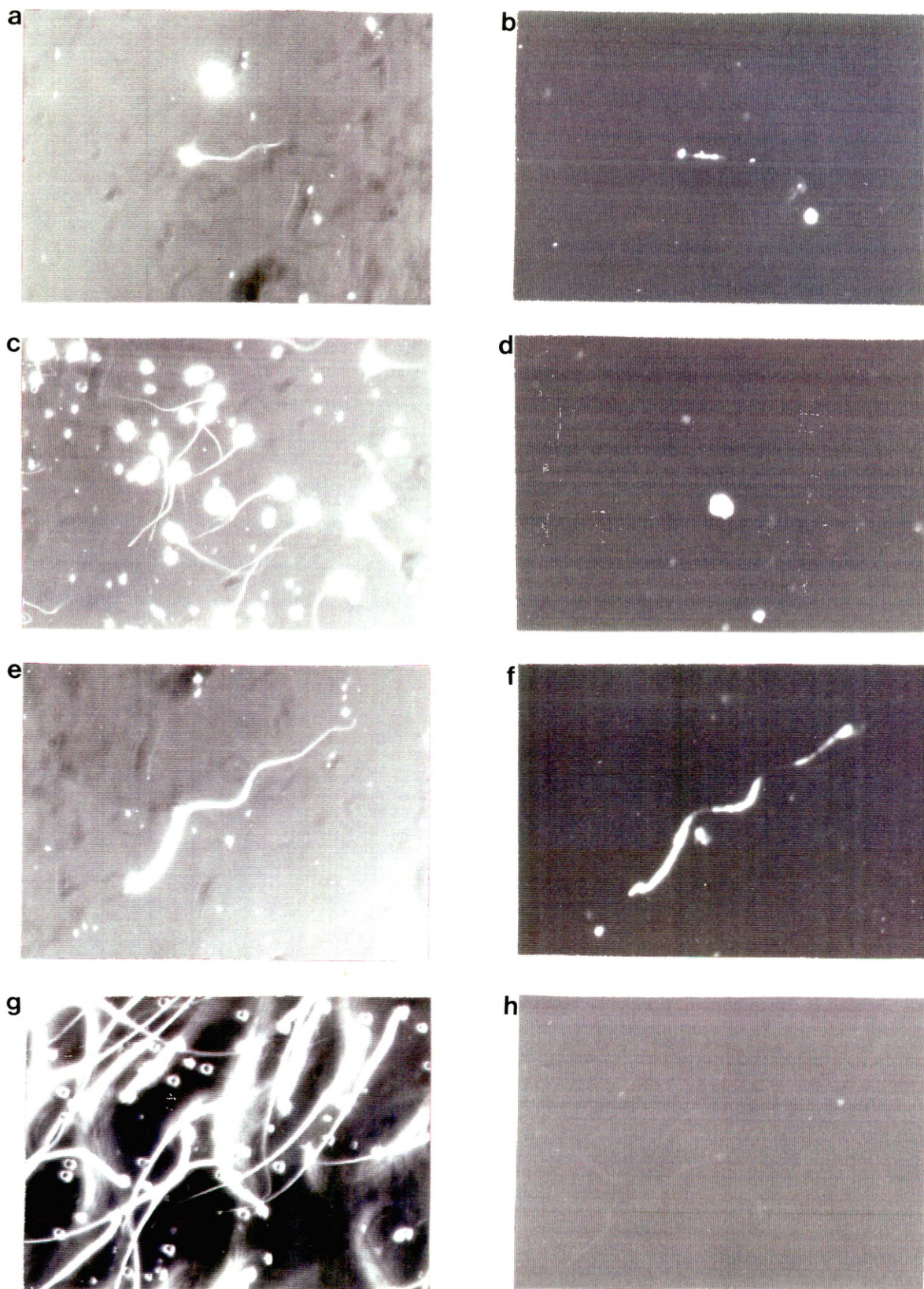


Figure 48. Photomicrographs of human(a,b) and gerbil(e,f) spermatozoa recovered from the chicken vagina by lavage 10 minutes following intravaginal insemination, and stained with FITC conjugated rabbit antichickens IgG. Photographs were taken using phase contrast(a,e) and epifluorescence(b,f) microscopy. Also shown are identically stained non-inseminated human(c,d) and gerbil(g,h) control spermatozoa.X400.

3.5.4.2. Correlation Between Sperm Live/Dead Status and Chicken Immunoglobulin Binding in the Vagina

Propidium iodide was observed to progressively permeate spermatozoa held under the coverslip, when used at a concentration of 20 $\mu\text{g/ml}$ or greater (Table 12) although spermatozoa held in a suspension containing 20 $\mu\text{g/ml}$ PI for up to 30 minutes appeared unaffected (11.7% of an average of 618 spermatozoa examined from 2 experiments staining dead). This effect was not observed at a concentration of 2 $\mu\text{g/ml}$, which was subsequently employed as the working concentration. At this concentration, propidium iodide was found to perform similarly to nigrosin and eosin, as a vital stain (see 2.6.3.2.1. for protocol).

Using propidium iodide (see 2.6.3.2.2.), $62.9 \pm 1.7\%$ (1281 spermatozoa examined, from 9 hens) of chicken spermatozoa recovered from the chicken vagina 20 minutes after insemination were stained, and $82.1 \pm 2.1\%$ of these had immunoglobulin bound to their surface (Figure 49). In contrast, only $7.7 \pm 3.4\%$ of spermatozoa excluding propidium iodide had chicken immunoglobulin bound to their surface.

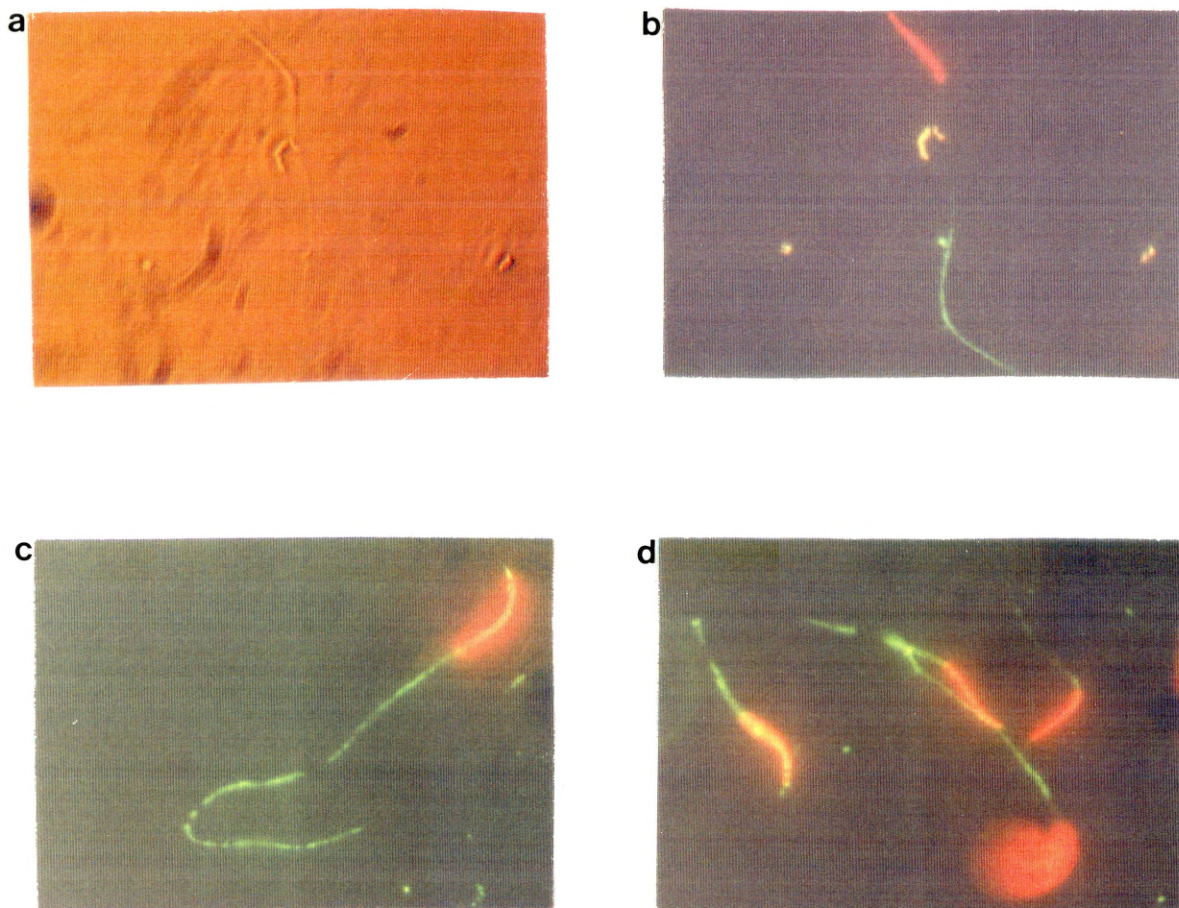


Figure 49. Photomicrographs of chicken spermatozoa recovered from the chicken vagina by lavage 20 minutes following intravaginal insemination, and stained with FITC conjugated rabbit antichickens IgG and Propidium Iodide. Photographs were taken using phase contrast(a) and epifluorescence(b,c,d) microscopy.X1000.

| [Propidium iodide] (ug/ml) | 0 min | 5 min | 10 min |
|----------------------------|-------|-------|--------|
| 20 | 10.2 | 35.3 | 52.5 |
| 2 | 10.5 | 11.3 | 9.5 |
| nigrosin & eosin | 9.4 | 9.9 | 9.4 |

Table 12. Live/dead status of chicken spermatozoa held under coverslips assessed by permeability to eosin or propidium iodide. Values are mean percentages of spermatozoa staining dead, from 2 experiments. An average of 748 spermatozoa were assessed per time under the coverslip, per stain.

3.5.4.3. Chicken Immunoglobulin Binding In-Vivo to Chicken Spermatozoa Recovered from the Anterior Oviduct Following Intravaginal and Intrauterine Insemination

Only $1.4 \pm 0.6\%$ (1516 spermatozoa examined, from 21 hens) of intravaginally inseminated spermatozoa subsequently recovered from the uterovaginal junction 48 hours following insemination had immunoglobulin bound to their surface (Figure 50) (see 2.6.3.5., 2.6.3.1. for protocol). Spermatozoa recovered from the infundibular mucosa (271 spermatozoa examined, from 12 hens) did not display immunoglobulin on their surface. Similar results were obtained after intrauterine insemination of spermatozoa (see 2.6.3.5., 2.6.3.1. for protocol). Only $1.3 \pm 2.1\%$ (1312 spermatozoa examined, from 10 hens) of such spermatozoa recovered from the uterovaginal junction and $0.1 \pm 0.8\%$ (983 spermatozoa examined, from 10 hens) of

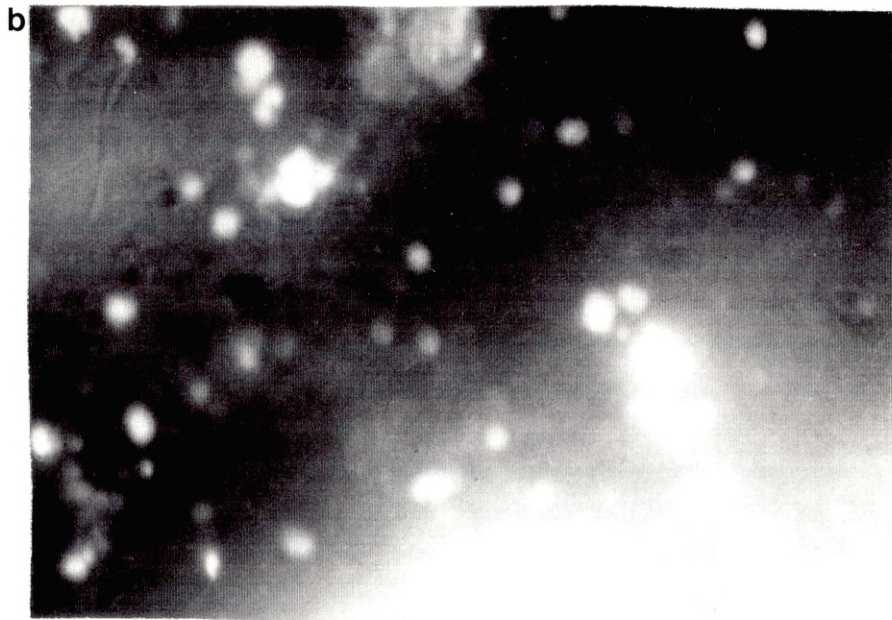
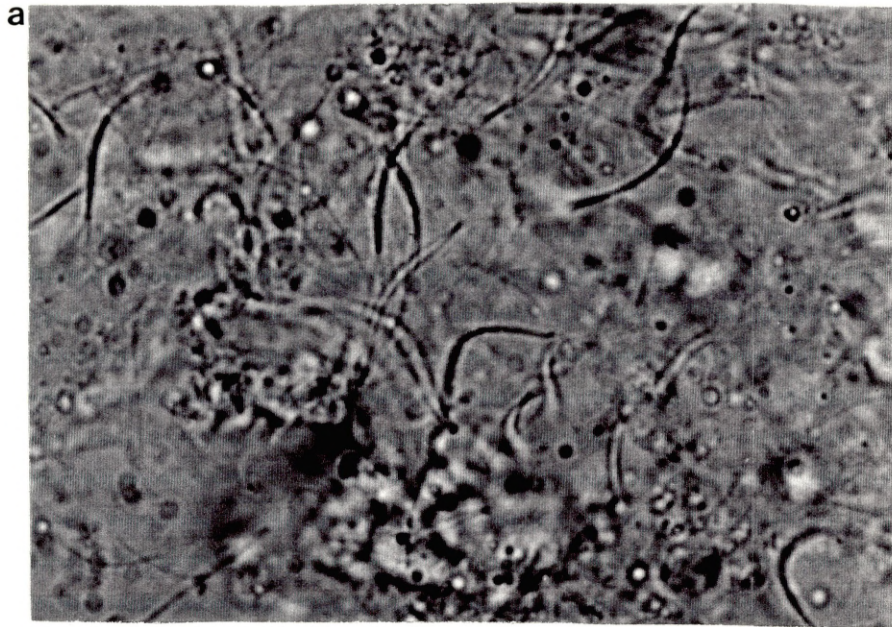


Figure 50. Photomicrographs of chicken spermatozoa recovered from the uterovaginal junction 48 hours following intravaginal insemination, and stained with FITC conjugated rabbit antichickn IgG. Photographs were taken using phase contrast(a) and epifluorescence(b) microscopy.X1000.

those recovered from the infundibular mucosa 3 hours after insemination displayed immunoglobulin on their surface.

3.5.4.4. Sperm Access to the Newly Ovulated Egg In-Vivo.
Chicken Immunoglobulin Binding and Live/Dead
Status Following In-Vitro Incubation with
Vaginal Mucosa

The percentage of spermatozoa with immunoglobulin bound to their surface was significantly greater ($P < 0.001$) following in-vitro incubation with vaginal mucosa, than after incubation in M.E.M. alone (see 2.6.4., 2.6.3.1. for protocol) (Table 13).

The percentages of spermatozoa staining dead (see 2.6.4., 2.3.1.2.3. for protocol) from infundibular and control incubations did not differ significantly ($P > 0.1$), but were both significantly lower than that from the vaginal incubation ($P < 0.001$).

| Sperm incubation | Chicken Immunoglobulin binding | | Live/dead staining | |
|-----------------------|--------------------------------|---------------------|--------------------|-----------------------|
| | No. sperm examined | % sperm binding IgG | No. sperm examined | % sperm staining dead |
| control (M.E.M. only) | 1200 | 3.0±1.0 | 1200 | 14.4±3.1 |
| vaginal mucosa | 1000 | 64.3±5.1 | 1500 | 49.5±1.8 |
| infundibular mucosa | - | - | 1500 | 20.7±1.8 |

Table 13. Chicken Immunoglobulin binding and live/dead status of chicken spermatozoa following a 60 minute incubation with vaginal and infundibular mucosa in-vitro. Values are mean \pm s.e.m. of data obtained from 5 experiments.

Access to the newly ovulated egg in-vivo (see 2.6.4., 2.3.1.2.5. for protocol) did not differ significantly between control spermatozoa and spermatozoa previously incubated with vaginal mucosa ($P>0.1$), with 1.1 ± 0.4 control spermatozoa/5.5 mm² of outer perivitelline layer, compared with 1.1 ± 0.3 for spermatozoa previously incubated with vaginal mucosa (from 23 and 19 eggs respectively).

3. 6. Sperm-Egg Interaction

3. 6. 1. Sperm Surface Changes Occurring During Transport Through the Oviduct

Spermatozoa recovered from the uterovaginal junction and infundibulum following intravaginal insemination (see 2. 7. 1.) showed similar staining to non-inseminated controls when exposed to antiserum raised against chicken seminal plasma proteins (see 2. 7. 1., 2. 2. 1. 2. for protocol), exhibiting intense uniform staining along their entire length. Staining intensities varied among samples due to varying numbers of spermatozoa and the presence of variable amounts of oviducal tissue, which also stained to a lesser degree.

Differential staining was observed on fixed and unfixed spermatozoa recovered from the oviduct and exposed to FITC-conjugated WGA (Figure 52) (see 2. 7. 1., 2. 2. 1. 3. for protocol). Fixed spermatozoa recovered from both oviducal regions and non-inseminated controls generally exhibited intense uniform staining along their entire length. However, $64.5 \pm 8.3\%$ (576 spermatozoa examined, from 10 hens) of unfixed spermatozoa recovered from the uterovaginal junction, and $72.4 \pm 9.3\%$ (171 spermatozoa examined, from 9 hens) of those recovered from the infundibulum (Figure 51) exhibited sparse, patchy staining along their entire length. These percentages were significantly greater ($P < 0.01$) than that for

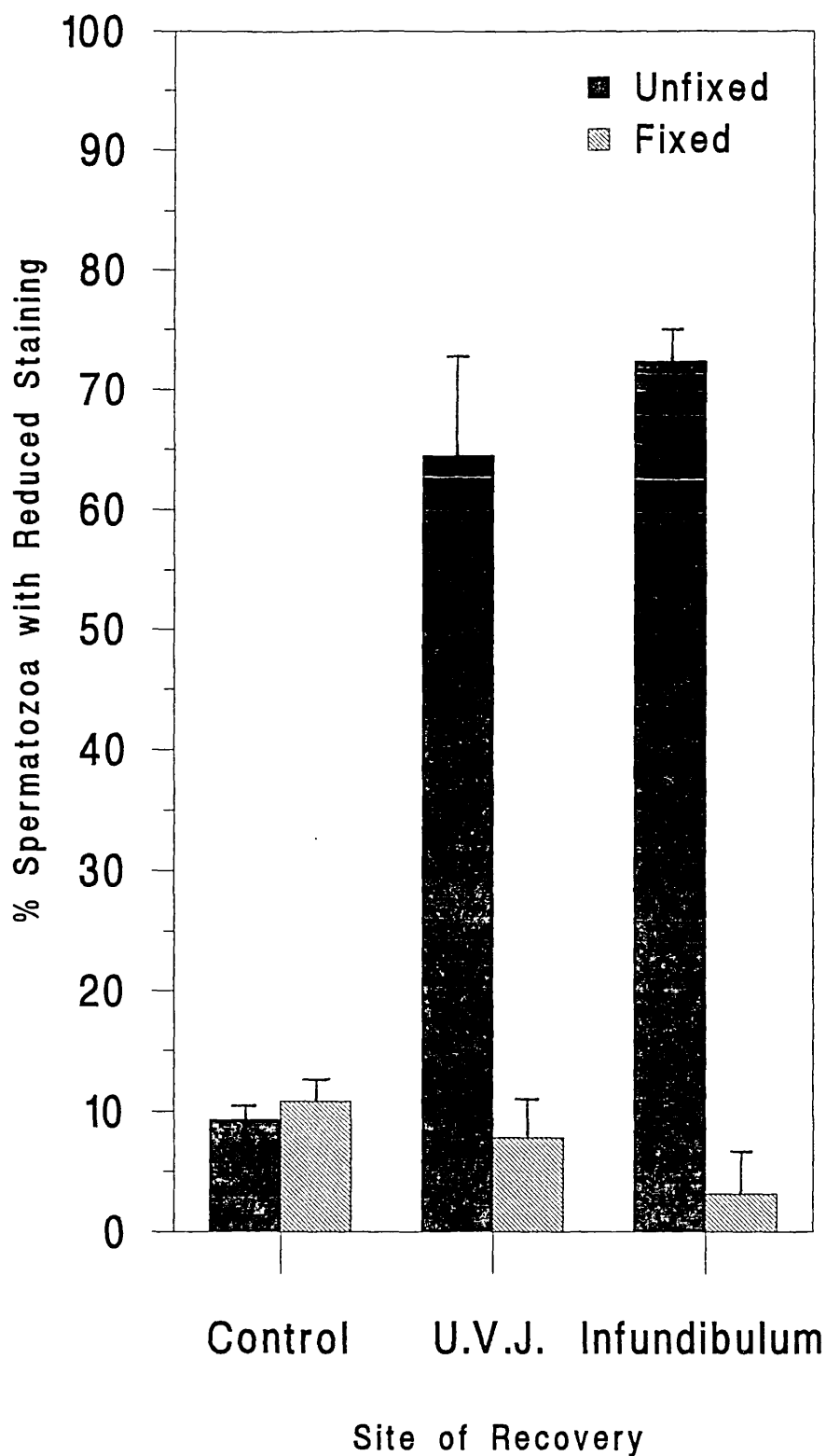


Figure 51. Reduced WGA staining of fixed and unfixed chicken spermatozoa recovered from the U.V.J. and Infundibulum following multiple I.V. insemination. Values are mean \pm s.e.m. from an average of 7 hens per oviducal region.

non-inseminated controls ($9.3 \pm 1.1\%$ of 1300 spermatozoa examined), but not significantly different from each other ($P > 0.1$). All unfixed control spermatozoa reacted with the lectin, but $5.2 \pm 6.8\%$ of those recovered from the uterovaginal junction, and $0.5 \pm 2.6\%$ of those recovered from the infundibulum failed to do so. The percentages of unfixed spermatozoa recovered by vaginal lavage 1 or 5 hours following insemination exhibiting reduced staining with FITC conjugated WGA (see 2.7.2., 2.2.1.3.) did not differ significantly from each other or from their respective controls ($P > 0.1$) (Table 14).

| Time of Recovery following insemination | No. hens used | No. sperm examined | % sperm with reduced staining |
|-----------------------------------------|---------------|--------------------|-------------------------------|
| 1 hr (control) | - | 1000 | 12.6 ± 0.7 |
| 1 hr | 4 | 907 | 14.2 ± 1.8 |
| 5 hr (control) | - | 1000 | 15.1 ± 1.0 |
| 5 hr | 3 | 457 | 11.4 ± 2.1 |

Table 14. Staining of spermatozoa recovered from the chicken vagina 1 or 5 hours following intravaginal insemination, or non-inseminated control spermatozoa with FITC-conjugated WGA. Values are mean \pm s.e.m..

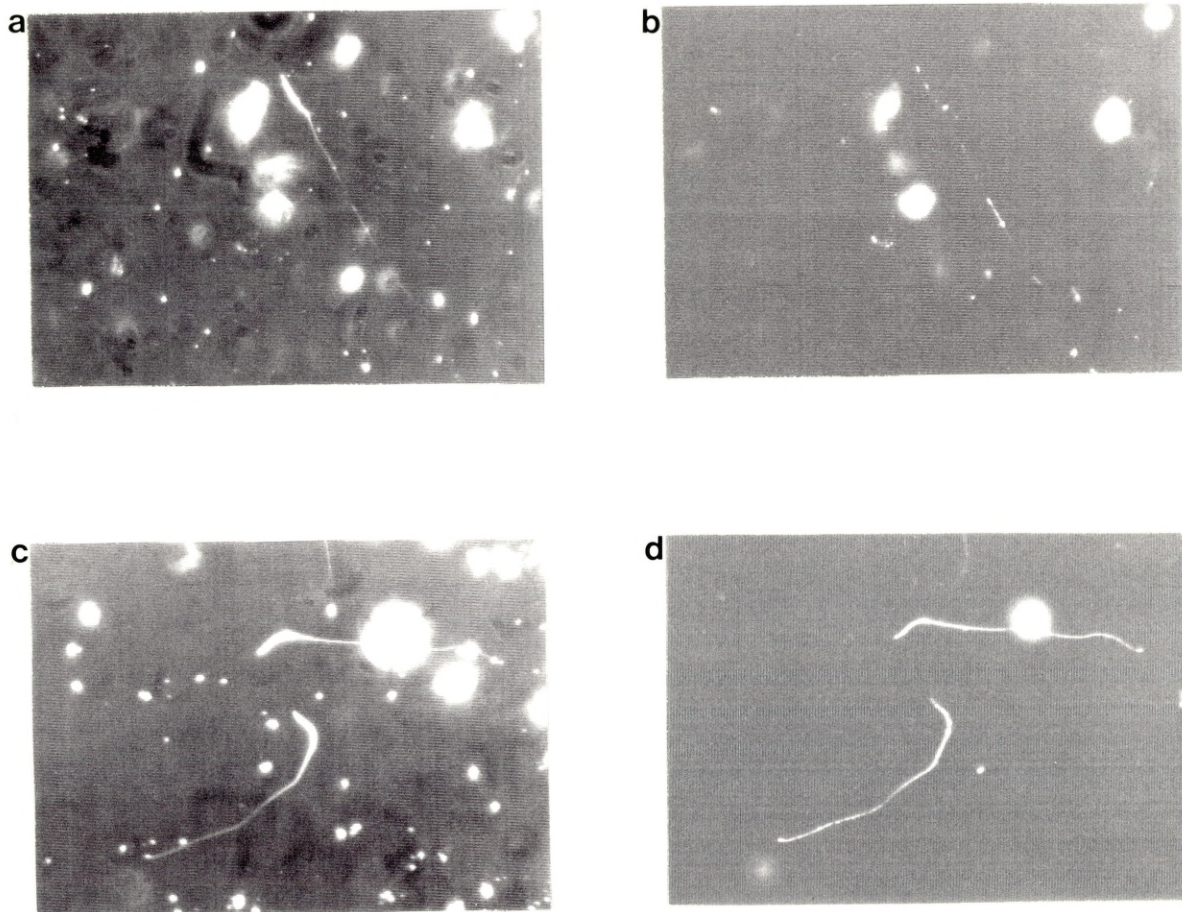


Figure 52. Photomicrographs of a typical unfixed spermatozoon recovered from the infundibulum prior to ovulation and 48 hours following intravaginal insemination(a,b), and non-inseminated control spermatozoa(c,d), stained with FITC conjugated WGA. Photographs were taken using phase contrast(a,c) and epifluorescence(b,d) microscopy.X400.

3. 6. 2. Effect of Ovarian Pocket Fluid and Inner Perivitelline Layer on the Sperm Surface and Live/Dead Status In-Vitro

Ovarian pocket fluid (OPF) elicited a definite change in sperm surface antigenicity (see 2.7.3.1., 2.7.3.2., 2.2.1.2. for protocol). Spermatozoa exposed to OPF (2800 spermatozoa examined from 5 experiments, using 14 different OPFs) exhibited greater variability in staining after exposure to antiserum raised against chicken seminal plasma proteins, than observed on control spermatozoa, which generally exhibited intense uniform staining along their entire length (1600 spermatozoa examined, from 5 experiments). Staining intensity was generally enhanced, particularly at the head and midpiece regions (Figure 53 (30 second exposure for epifluorescence photographs)).

Spermatozoa exposed to OPF (1000 spermatozoa examined, from 3 experiments, using 5 OPFs) exhibited similar staining to control spermatozoa (800 spermatozoa examined, from 3 experiments) after exposure to FITC conjugated WGA (see 2.7.3.2., 2.2.1.3. for protocol), with intense uniform staining along their entire length and >80% of spermatozoa agglutinated.

Spermatozoa incubated with chicken inner perivitelline layer in the presence or absence of OPF exhibited similar immunofluorescent and lectin staining (see 2.7.3.1.,

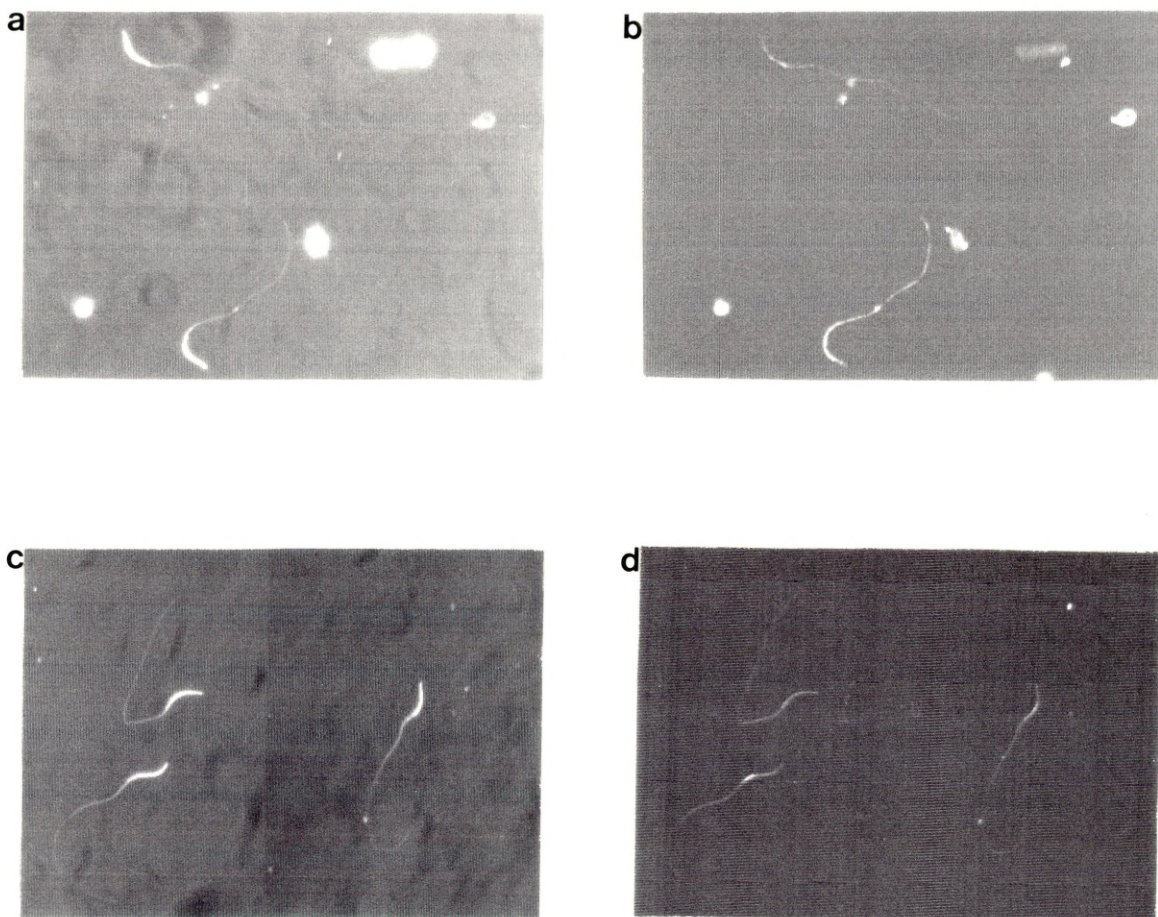


Figure 53. Photomicrographs of spermatozoa exposed to ovarian pocket fluid diluted 1:1 with M.E.M. for 60 minutes (a,b), and control spermatozoa (c,d), stained with rabbit antiserum raised against ejaculated chicken seminal plasma proteins, and FITC conjugated goat antirabbit IgG. Photographs were taken using phase contrast(a,c) and epifluorescence(b,d) microscopy.X400.

2.7.3.2., 2.2.1.2., 2.2.1.3. for protocols)
(1800 spermatozoa examined for each stain, from
9 experiments) to that observed in the absence of inner
perivitelline layer.

Spermatozoa incubated with the animal pole of the inner
perivitelline layer in the presence or absence of OPF
(600 spermatozoa examined for each stain, from
3 experiments) exhibited similar immunofluorescent and
lectin staining intensities to spermatozoa exposed to the
vegetal pole. This was also the case for spermatozoa
incubated with inner perivitelline layer from early and
intermediate follicular eggs (600 spermatozoa examined for
each stain, from 3 experiments), and spermatozoa incubated
with whole perivitelline layer from laid eggs
(800 spermatozoa examined for each stain, from
4 experiments).

The percentage of spermatozoa excluding eosin after a
30 minute exposure to OPF diluted 1:1 with M.E.M. at
40 °C (see 2.8.1., 2.3.1.2.3. for protocol), did not
differ significantly from control spermatozoa ($P>0.1$)
(Table 15). However, spermatozoa exposed to OPF were
significantly less motile (see 2.8.1., 2.3.1.2.1. for
protocol) after a 10 minute incubation at 40 °C than
control spermatozoa ($P<0.001$).

| Incubation | Motility (%[OD]m) | % Sperm excluding eosin |
|-----------------------------|--------------------|-------------------------|
| sperm (control) | 59.4±0.9 | 94.2±3.0 |
| sperm + OPF | 20.6±3.6 | 94.1±1.5 |
| sperm + Inner P.L. | 55.1±2.3 | 93.4±1.8 |
| sperm + Inner P.L. + OPF | 13.3±2.9 | 94.3±2.1 |

Table 15. Effect of OPF and inner perivitelline layer on sperm motility and live/dead status. Values are mean \pm s.e.m. of data obtained after a 30 minute incubation at 40 °C in the absence of inner perivitelline layer or 5 minutes after addition of inner perivitelline layer (eosin exclusion), or 10 minutes following the addition of inner perivitelline layer (motility). A total of 1200 spermatozoa per incubation were assessed for eosin exclusion.

The percentage of spermatozoa excluding eosin after a prolonged 120 minute exposure to OPF did not differ significantly from control spermatozoa or from their respective percentages after 30 minutes incubation ($P>0.1$) (Table 16).

Incubations carried out in the presence of inner perivitelline layer produced similar results. The percentages of spermatozoa excluding eosin following a 5 minute incubation in the presence or absence of OPF did not differ significantly from each other, from their respective values after a prolonged 90 minute incubation, or from their respective values in the absence of inner perivitelline layer ($P>0.1$).

The motility of spermatozoa incubated with OPF was significantly lower than that of control spermatozoa ($P < 0.001$). Using light microscopy, a reduction in sperm motility was observed almost immediately after addition to medium containing OPF, whereas spermatozoa incubated in the absence of OPF maintained vigorous motility. However, motility values did not differ significantly from those obtained in the absence of inner perivitelline layer ($P > 0.1$).

| Time of Incubation (min) | spermatozoa (control) | spermatozoa + OPF | spermatozoa + inner P. L. | spermatozoa + OPF + inner P. L. |
|--------------------------|-----------------------|-------------------|---------------------------|---------------------------------|
| 5 | - | - | 93.4 \pm 1.5 | 94.3 \pm 1.8 |
| 30 | 94.2 \pm 3.1 | 94.0 \pm 1.7 | - | - |
| 90 | - | - | 91.3 \pm 2.4 | 91.9 \pm 1.9 |
| 120 | 91.0 \pm 2.6 | 92.5 \pm 0.8 | - | - |

Table 16. Effect of OPF and inner perivitelline layer on sperm live/dead status in-vitro. Values are mean \pm s.e.m. percentages of spermatozoa excluding eosin during a 120 minute incubation at 40 °C in the presence or absence of OPF, or a 90 minute incubation in the presence or absence of inner perivitelline layer, subsequent to a 30 minute incubation in the absence of inner perivitelline layer. Data were obtained from 4 experiments.

3.6.3. Interaction of Spermatozoa with the Perivitelline Layer of the Egg In-Vitro

Incubation of spermatozoa with inner perivitelline layer from newly ovulated eggs (see 2.8.1. for protocol) resulted in the appearance of dark circular areas against the otherwise brightly fluorescing membrane after staining with FITC conjugated WGA (Figure 54). Phase contrast microscopy showed that many of these areas comprised holes penetrating both the inner perivitelline layer and plasma membrane, and sperm heads, in many cases apparently lacking acrosomal caps were often observed in association with them. Inner perivitelline layer from newly ovulated eggs, incubated in the absence of spermatozoa, showed no such holes, which were presumed to be areas of hydrolysis produced by sperm acrosome reactions, as described by Koyanagi et al. (1988), using phase contrast microscopy.

Incubation of spermatozoa exposed to OPF with inner perivitelline layer resulted in a significantly lower concentration of points of hydrolysis on the membrane than was produced in the absence of OPF ($P < 0.001$) (Table 17). Similarly, the concentration of points of hydrolysis produced on inner perivitelline layer from newly ovulated eggs was significantly greater than that produced on whole perivitelline layer from laid eggs ($P < 0.001$). However, results obtained from the incubation of spermatozoa with inner perivitelline layer from early follicular oocytes and inner perivitelline layer from the animal pole of

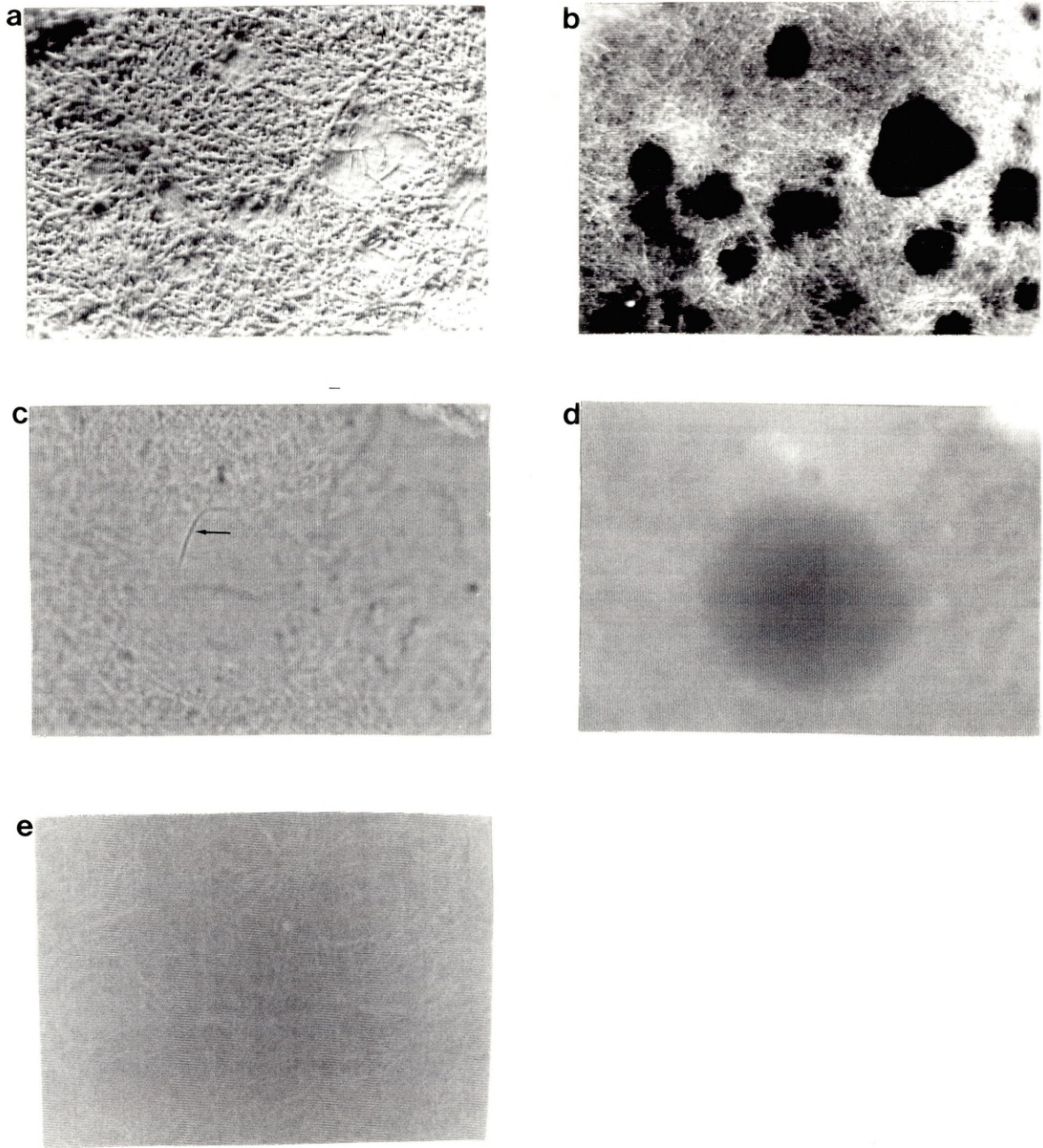


Figure 54. Photomicrographs of inner perivitelline layer from newly ovulated chicken eggs following incubation with chicken spermatozoa in-vitro(a,b,c,d), stained with FITC conjugated WGA and showing sites of acrosome reactions. Also shown is control inner perivitelline layer not incubated with spermatozoa(e). Photographs were taken using phase contrast(a,c) and epifluorescence(b,d,e) microscopy. X400(a,b,e); X1000(c,d).

newly ovulated eggs did not differ significantly from those obtained using inner perivitelline layer from the vegetal pole of newly ovulated eggs ($P>0.1$).

| Perivitelline Layer | \pm OPF | No. points of hydrolysis/field | No. membranes examined |
|---------------------------------|-----------|--------------------------------|------------------------|
| Inner (vegetal pole) | - | 85.0 \pm 3.3 | 16 |
| Inner (vegetal pole) | + | 5.2 \pm 1.2 | 5 |
| Inner (animal pole) | - | 86.0 \pm 4.2 | 3 |
| Inner (early follicular) | - | 80.9 \pm 0.7 | 3 |
| Inner (intermediate follicular) | - | 76.9 \pm 2.5 | 3 |
| Whole | - | 6.6 \pm 1.1 | 6 |

Table 17. Interaction of chicken spermatozoa with perivitelline layer. Values are mean \pm s.e.m. number of points of hydrolysis per whole microscopic field at X400 magnification. Twenty fields were assessed per membrane.

Chicken, turkey, guinea fowl and duck spermatozoa interacted with heterologous inner perivitelline layer (see 2.8.2.2., 2.8.1. for protocol). However, both chicken and guinea fowl spermatozoa demonstrated a greater interaction with homologous than heterologous inner perivitelline layer (Table 18). Chicken spermatozoa produced a greater concentration of points of hydrolysis on chicken inner perivitelline layer than any of the other spermatozoa used, and considerably outperformed guinea fowl spermatozoa on guinea fowl inner perivitelline layer.

| Spermatozoa | Inner perivitelline layer | No. points of hydrolysis/field | No. membranes examined |
|-------------|---------------------------|--------------------------------|------------------------|
| chicken | chicken | 91.4 | 2 |
| chicken | guinea fowl | 60.7 | 2 |
| guinea fowl | guinea fowl | 3.2 | 2 |
| guinea fowl | chicken | 0.6 | 2 |
| duck | chicken | 0.2 | 2 |
| duck | guinea fowl | 0.2 | 2 |
| turkey | chicken | 44.9±10.6 | 7 |

Table 18. Interaction of chicken, guinea fowl, duck and turkey spermatozoa with chicken and guinea fowl inner perivitelline layer in-vitro. Values are the mean number of points of hydrolysis per whole microscopic field at X400 magnification. Twenty fields were assessed per membrane.

The concentrations of points of hydrolysis produced on chicken inner perivitelline layer by chicken or turkey spermatozoa following absorption with sonicated preparations of chicken inner or whole perivitelline layer (see 2.8.2.3., 2.8.1. for protocol) were not significantly different from each other ($P>0.1$) (Table 19), but differed significantly from the concentrations produced in the absence of the absorption step ($P<0.001$). Chicken spermatozoa incubated with chicken inner perivitelline layer after absorption with sonicated whole chicken perivitelline layer produced a concentration of points of hydrolysis not significantly different from that obtained following absorption with sonicated whole perivitelline

layer from laid turkey eggs ($P>0.1$), but significantly different from that obtained following absorption with sonicated whole perivitelline layer from laid quail eggs ($P<0.02$) (Table 19) (see 2.8.2.4., 2.8.1. for protocol).

The protein concentrations of sonicated chicken inner perivitelline layer and chicken, turkey and quail whole perivitelline layer preparations were 6.4, 7.3, 7.7 and 6.9 mg/ml respectively, as determined spectrophotometrically at 280nm (see 2.8.2.3. for protocol). Spermatozoa remained vigorously motile following absorption with sonicated preparations, as determined microscopically.

| Spermatozoa | Sonicated perivitelline layer | No. points of hydrolysis/field | No. membranes examined |
|-------------|-------------------------------|--------------------------------|------------------------|
| chicken | - | 85.0 \pm 3.3 | 16 |
| chicken | chicken inner | 3.1 \pm 1.3 | 4 |
| turkey | - | 44.9 \pm 10.6 | 7 |
| turkey | chicken inner | 1.9 \pm 0.6 | 3 |
| chicken | chicken whole | 3.6 \pm 1.0 | 7 |
| turkey | chicken whole | 1.4 \pm 0.8 | 3 |
| chicken | turkey whole | 3.2 \pm 1.3 | 3 |
| chicken | quail whole | 22.7 \pm 9.4 | 3 |

Table 19. Interaction of chicken and turkey spermatozoa with chicken inner perivitelline layer in-vitro following absorption with sonicated perivitelline layer preparations. Values are the mean \pm s.e.m. number of points of hydrolysis per whole microscopic field at X400 magnification. Twenty fields were assessed per membrane.

3.6.3.1. Electrophoretic Profiles of Chicken Inner Perivitelline Layer and Chicken, Quail and Turkey Whole Perivitelline Layer, following Incubation with Chicken Spermatozoa In-Vitro

The electrophoretic profile of sonicated chicken inner perivitelline layer (see 2.8.2.4., 2.2.2.2. for protocol) contained approximately 20 protein bands, ranging in molecular weight from 10 kd to >200 kd. Prominent bands were observed at 44, 66, 180 and >200 kd (Figure 55 (negative photographic image)). Following absorption with chicken spermatozoa (see 2.8.2.4 for protocol) no protein bands were observed at 44 or 180 kd, and the band at >200 kd exhibited considerably reduced staining intensity. Other high molecular weight proteins and a weakly stained band at 150 kd also exhibited reduced staining intensities, but the prominent band at 66 kd appeared relatively unaffected by absorption.

The electrophoretic profile of sonicated chicken whole perivitelline layer contained approximately 25 protein bands, ranging in molecular weight from 10 kd to >200 kd (Figure 56). Strongly stained bands at 13, 44 and 80 kd appeared relatively unchanged in the absorbed profile. However, the band at >200 kd exhibited a considerably reduced staining intensity, with the band at 180 kd absent from the absorbed profile. Other bands at 16 and 48 kd and all high molecular weight bands showed a degree

of reduction in staining intensity on the absorbed profile.

The electrophoretic profile of sonicated turkey whole perivitelline layer appeared similar to that of the chicken. Differences included the presence of an extra band at 75 kD in the turkey profile, the slightly lower molecular weight (15.5 kD) of a protein apparently equivalent to the 16 kD protein on the chicken profile, and slight differences in banding pattern and molecular weights of high molecular weight proteins (Figure 56). The turkey profile contained approximately 20 protein bands with a similar molecular weight range to that of chicken. Bands at 13, 15.5, 70, 75 and 80 kD appeared relatively unchanged in the absorbed profile. However, the bands at 48 and 66 kD showed a slight reduction in staining intensity, with those at 44 and 50 kD considerably weaker. Several high molecular weight bands were absent from the absorbed profile.

The sonicated quail whole perivitelline layer profile differed from that of chicken, with differences in banding pattern and molecular weights of high molecular weight proteins, and a considerably weaker 16 kD band in the quail profile (Figure 56). Approximately 20 protein bands were apparent, with those at 13 and 48 kD appearing relatively unchanged in the absorbed profile. However, bands at 66 and 80 kD showed a slight reduction in

staining intensity, and all bands above 80 kd were absent from the absorbed profile.

Staining intensities of protein bands on electrophoretic profiles of sonicated whole perivitelline layer from the animal and vegetal poles of laid chicken eggs (see 2.8.2.1., 2.2.2.2. for protocol) appeared similar (not shown).

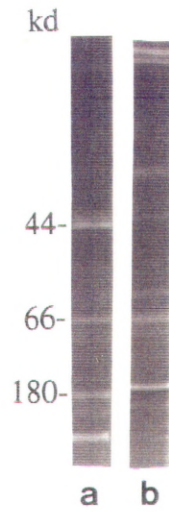


Figure 55. Silver stained electrophoretic profiles of sonicated preparations of chicken inner perivitelline layer prior to (a) and following (b) absorption with chicken spermatozoa.

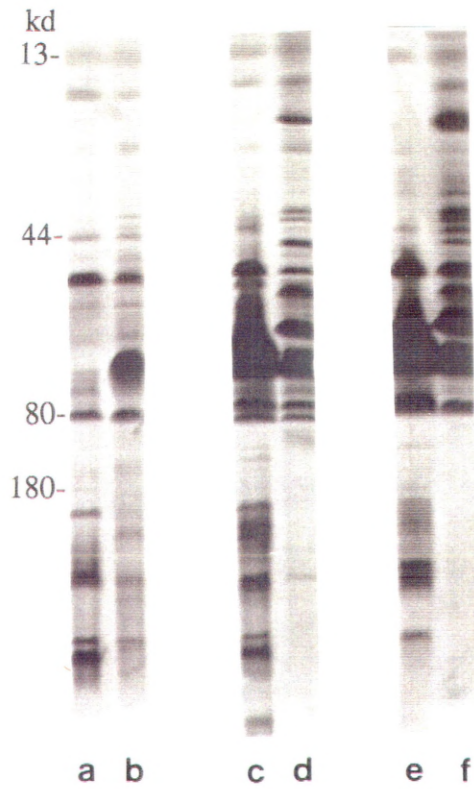


Figure 56. Silver stained electrophoretic profiles of sonicated preparations of chicken, turkey and quail whole perivitelline layer prior to (a,c,e respectively), and following (b,d,f respectively), absorption with chicken spermatozoa.

3. 7. Comparison of Sperm Surfaces and Seminal Plasmas from Different Avian Species

3. 7. 1. Cross-reactivity of Antiserum Raised Against Chicken Seminal Plasma Proteins, with Heterologous Avian Spermatozoa

Chicken spermatozoa stained with antiserum raised against chicken seminal plasma proteins, and FITC-conjugated goat antirabbit IgG (see 2.9.1., 2.2.1.2. for protocol) exhibited intense uniform fluorescence along their entire length (Figure 57 (30 second exposure for epifluorescence photographs)). Quail, pigeon and duck spermatozoa also exhibited uniform staining along their entire length, but of reduced intensity, more so on duck and quail spermatozoa. Turkey spermatozoa (see Figure 36) exhibited less uniform staining, mainly restricted to the head, midpiece and anterior to mid-flagellar regions (1200 spermatozoa were examined from each species, from 3 experiments).

3. 7. 2. Lectin Staining of Spermatozoa from Different Avian Species

FITC-conjugated WGA produced considerably more intense staining on chicken spermatozoa than on spermatozoa from any of the other species studied (Figure 58 (30 second exposure for epifluorescence photographs)). Unfixed and

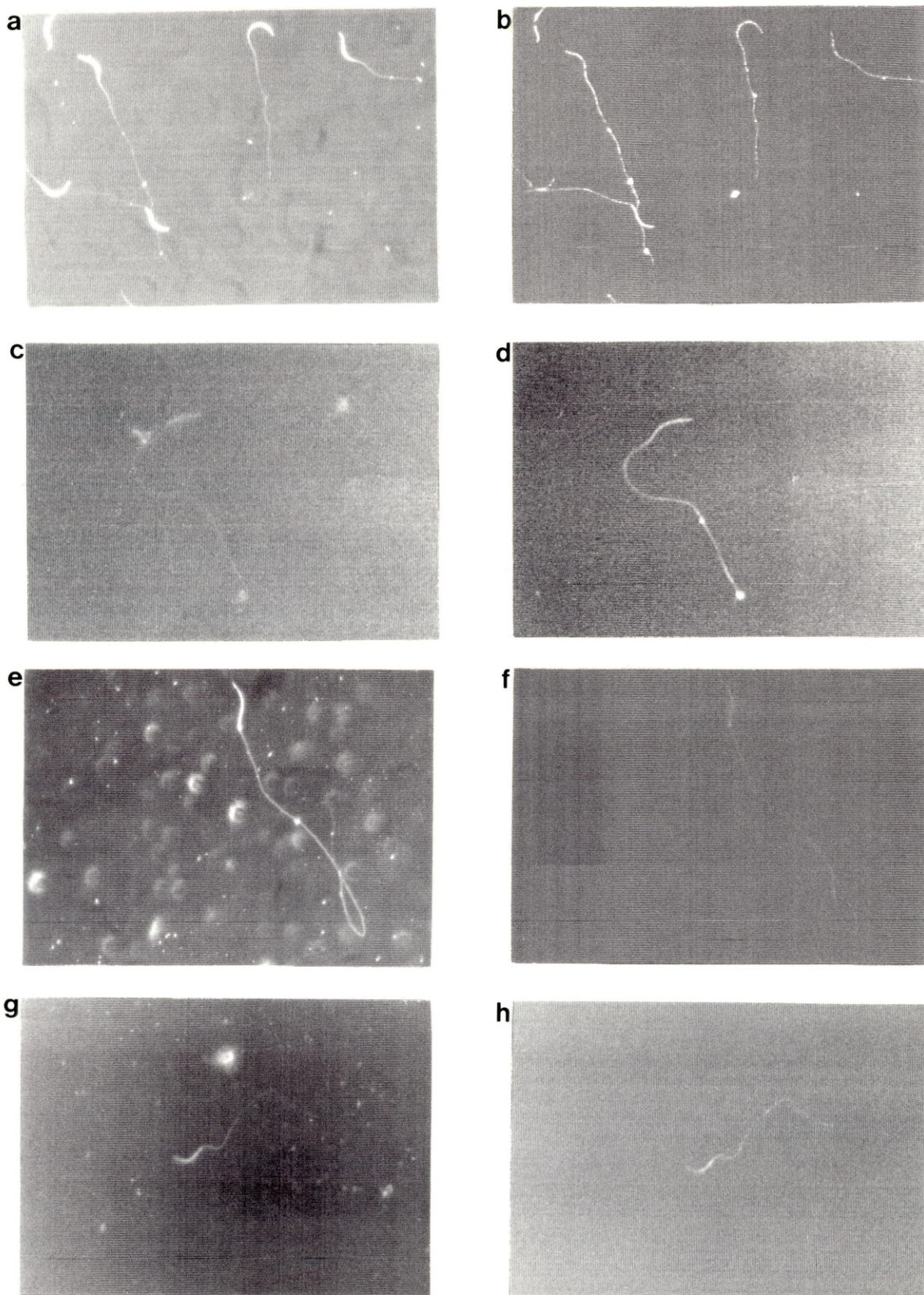


Figure 57. Photomicrographs of chicken(a,b), pigeon(c,d), quail(e,f) and duck(g,h) spermatozoa stained with rabbit antiserum raised against ejaculated chicken seminal plasma proteins, and FITC conjugated goat antirabbit IgG. Photographs were taken using phase contrast(a,c,e,g) and epifluorescence(b,d,f,h) microscopy.X400.

fixed chicken spermatozoa typically exhibited uniform staining along their entire length (see 2.9.1., 2.2.1.3. for protocol), and >80% were agglutinated. Sperm-sperm agglutination was mainly flagellar, and agglutinated spermatozoa generally exhibited more intense staining. Unfixed and fixed duck, turkey and pigeon spermatozoa also exhibited uniform staining along their entire length, although of lower intensity. For these 3 species, >50% of spermatozoa were agglutinated by the lectin, with sperm-sperm agglutination being mainly flagellar, and agglutinated spermatozoa generally staining more intensely. Agglutinations, as for chicken spermatozoa, were of variable size, ranging from around 10 up to several hundred spermatozoa.

Unfixed and fixed quail spermatozoa (1200 spermatozoa examined, from 3 experiments) exhibited patchy staining along their entire length of considerably lower intensity than that observed on chicken spermatozoa, with $34.2 \pm 1.1\%$ of unfixed and $37.3 \pm 7.3\%$ of fixed spermatozoa exhibiting more intense uniform staining of the head region. Some flagellar sperm-sperm agglutination (<10%) was evident (1200 non-agglutinated spermatozoa were examined from each species, from 3 experiments).

Unfixed and fixed chicken, turkey, pigeon and duck spermatozoa all showed similar staining intensities and distributions following exposure to FITC-conjugated ConA, exhibiting faint uniform fluorescence restricted to the

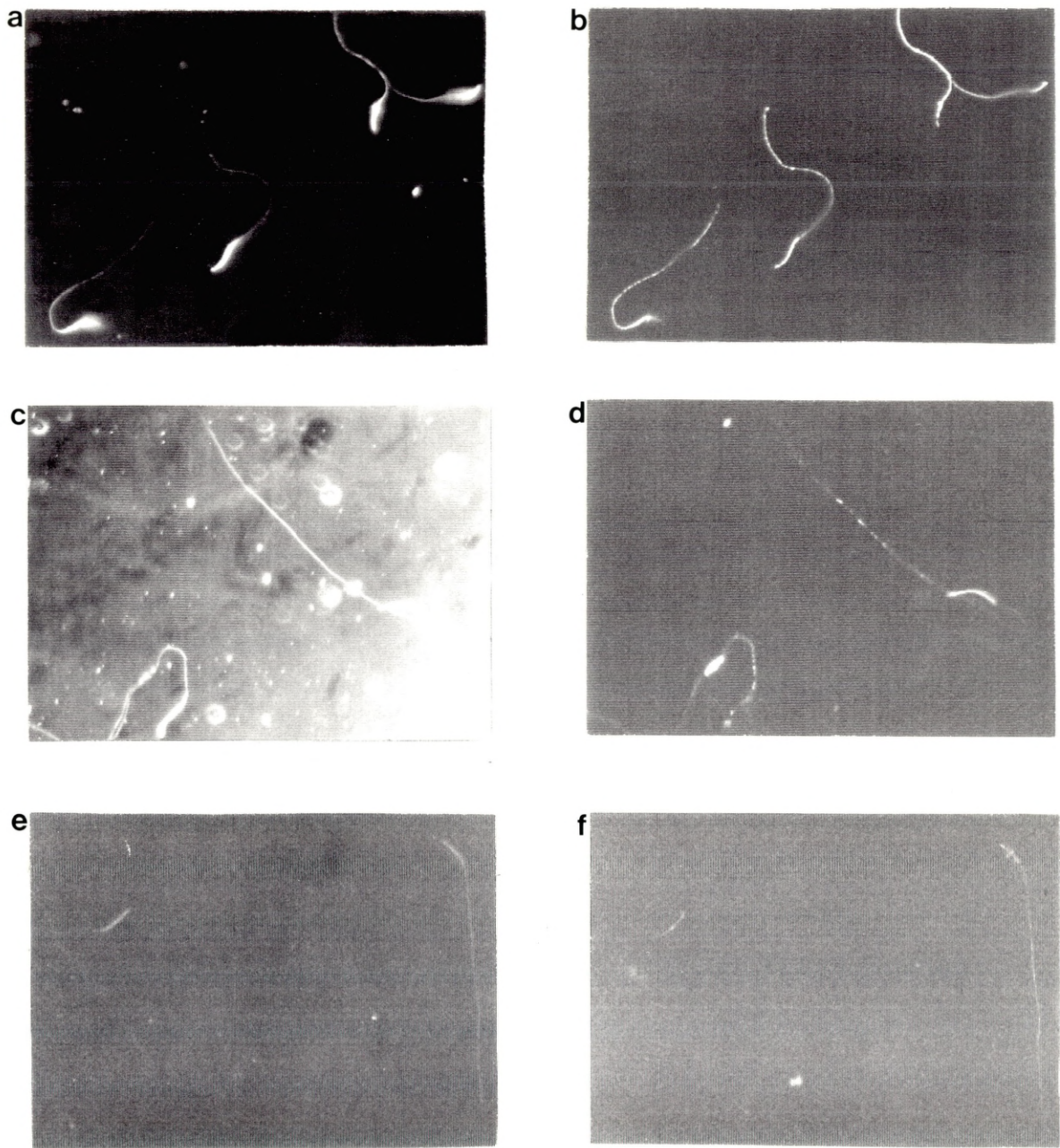


Figure 58. Photomicrographs of unfixed chicken(a,b), quail(c,d) and pigeon(e,f) spermatozoa stained with FITC conjugated WGA, taken using phase contrast(a,c,e) and epifluorescence(b,d,f) microscopy.X400.

head or head and midpiece regions (Figure 59 (30 second exposure for epifluorescence photographs)). Fixed and unfixed quail spermatozoa were stained uniformly along their entire length, with the posterior head region slightly more intensely stained (Figure 59). None of the spermatozoa studied were agglutinated by the lectin (1200 spermatozoa were examined from each species, from 3 experiments).

Unfixed and fixed quail spermatozoa were neither stained nor agglutinated by lectin from Solanum tuberosum. Unfixed and fixed chicken (Figure 59), turkey, duck and pigeon spermatozoa all showed similar staining, exhibiting variable patchy staining along their entire length. More than 50% of chicken, pigeon and turkey spermatozoa were agglutinated, whereas duck spermatozoa were not. Agglutination was flagellar, and was accompanied by enhanced staining (1200 spermatozoa were examined from each species, from 3 experiments).

Unfixed and fixed quail spermatozoa stained with FITC-conjugated lectin from Tetragonolobus purpureas exhibited variable patchy fluorescence along their entire length (Figure 59), with >30% agglutinated. Sperm-sperm agglutination was mainly flagellar, with agglutinated spermatozoa exhibiting more intense and uniform staining. Agglutinations were variable in size, but generally small, comprising less than 50 spermatozoa. Unfixed and fixed chicken, turkey, duck and pigeon spermatozoa were neither

stained nor agglutinated by the lectin (1200 spermatozoa were examined from each species, from 3 experiments).

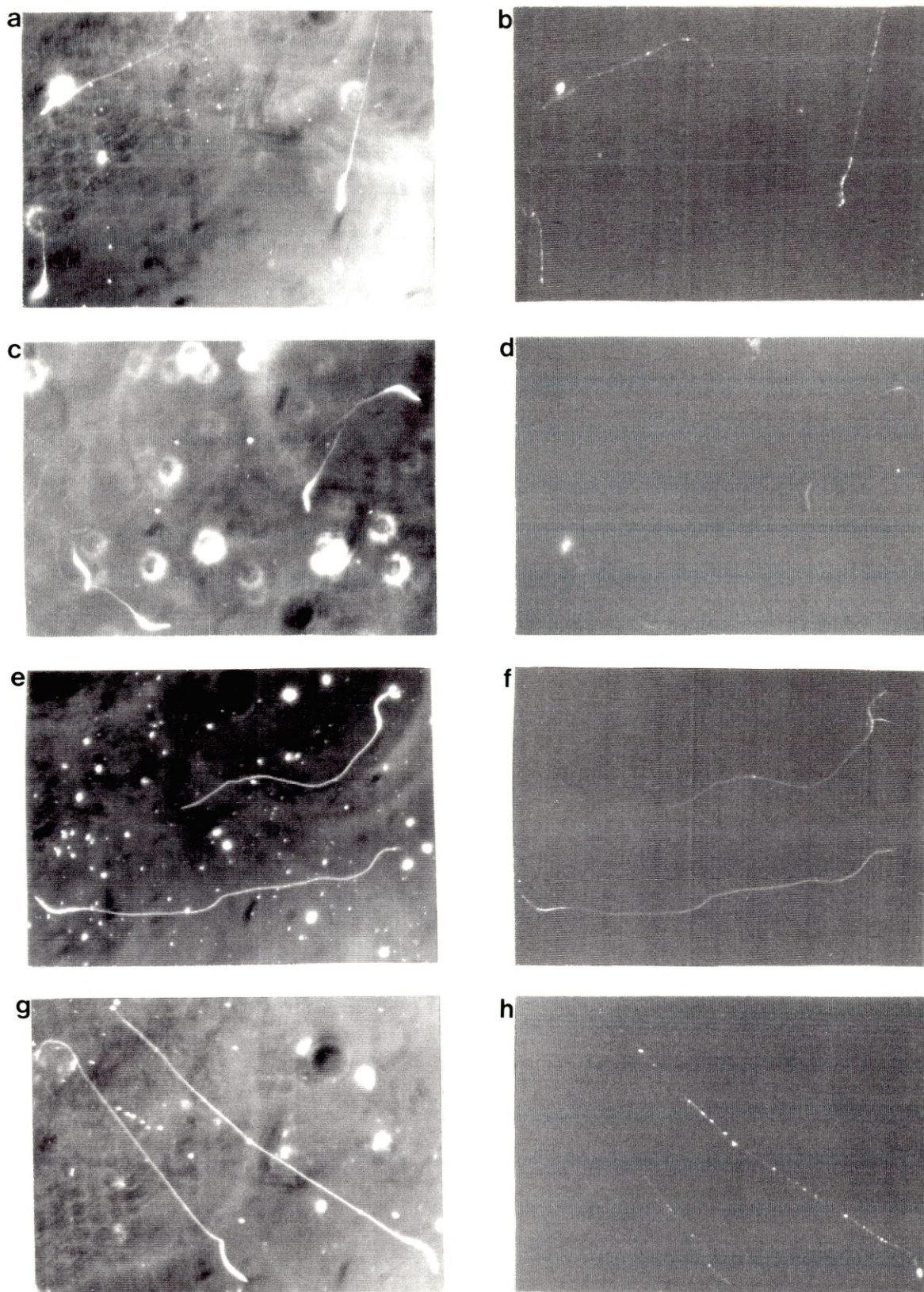


Figure 59. Photomicrographs of unfixed chicken(a,b,c,d) and quail(e,f,g,h) spermatozoa stained with FITC conjugated lectin from Solanum tuberosum(a,b), FITC conjugated Con A(c,d,e,f) and lectin from Tetragonolobus purpureas(g,h). Photographs were taken using phase contrast(a,c,e,g) and epifluorescence(b,d,f,h) microscopy.X400.

3. 7. 3. Electrophoretic Analysis of Seminal Plasmas from Different Avian Species

3. 7. 3. 1. Silver Stained Electrophoretic Profiles of Seminal Plasmas from Different Avian Species

Electrophoretic profiles of seminal plasmas (see 2.9.2., 2.2.2.2. for protocol) displayed between 45 and 60 protein bands, with the majority of bands being found between 10 and 100 kd (Figure 60). Species-specific banding patterns were observed, although bands were observed at 25, 35, 53, 58 and 66 kd in profiles from all species. Such bands were stained at varying intensities among species.

The dominant protein band in the chicken, turkey and duck profiles was at 66 kd, with another prominent band at 80 kd (around 75 kd for duck). In the quail profile, the protein band at 21 kd was most intensely stained, with other prominent bands at 10.5, 66 and 75 kd. There were no intensely stained bands in the pigeon seminal plasma profiles.

3. 7. 3. 2. Immunologically Stained Blotted Seminal Plasma Proteins From Different Avian Species

Antiserum raised against chicken seminal plasma proteins cross-reacted with seminal plasma proteins from all species studied (Figure 61) (see 2.9.2., 2.2.2.3. for

species, with 12 bands observed on the turkey profile, compared with 11 bands observed in the homologous (chicken) profile. Intensity of staining was comparable for the 2 profiles. Fewer bands were observed on quail (6), duck (8) and pigeon (4) profiles, with pigeon seminal plasma proteins exhibiting weaker staining than observed for other species.

The most intensely stained band in profiles from all species was at 66 kD, with bands at 13.5, 25, 43, 46 and 49 kD relatively intensely stained in chicken profiles (Figures 60, 61). This was also the case for bands at 43, 47, 58 and 60 kD on turkey; 12.5, 22, 53 and 75 kD on quail; and 23, 27 and 58 kD on duck profiles.

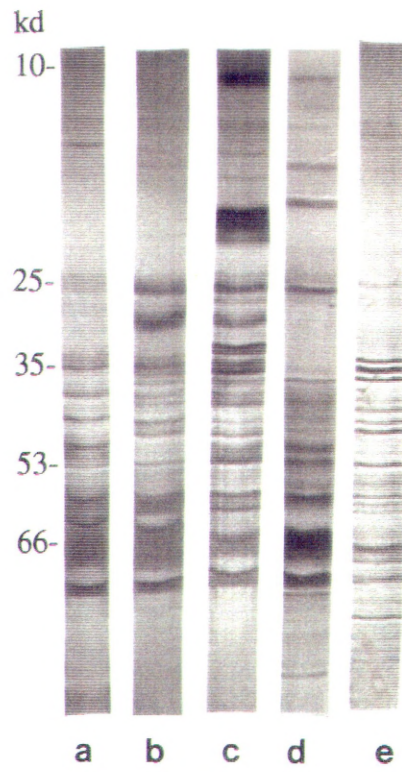


Figure 60. Silver stained electrophoretic profiles of chicken(a), turkey(b), quail(c), duck(d) and pigeon(e) seminal plasma proteins.

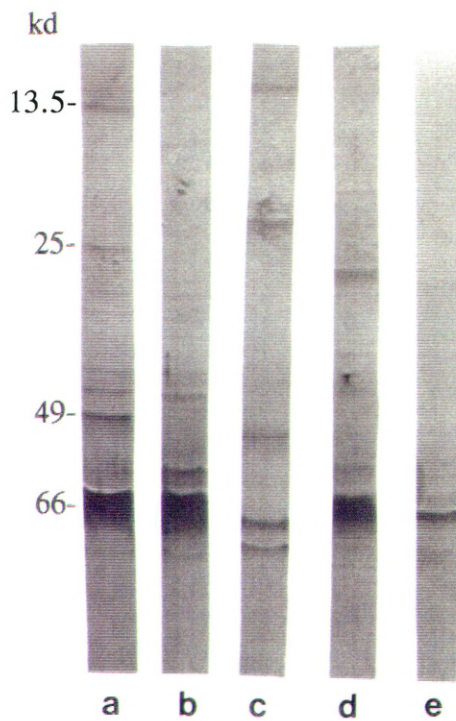


Figure 61. Western blot of chicken(a), turkey(b), quail(c), duck(d) and pigeon(e) seminal plasma proteins stained with rabbit antiserum raised against chicken ejaculated seminal plasma proteins, and HRP conjugated donkey antirabbit IgG.

CHAPTER 4. DISCUSSION

4. DISCUSSION

4. 1. Post-testicular Maturation

4. 1. 1. Seminal Plasma and Sperm Surface-Associated Proteins

Clear changes occurred in the protein composition of chicken seminal plasma between the anterior ductus deferens and the ejaculate, involving their relative concentrations in seminal plasma profiles, and their carbohydrate and antigenic character (see 3.1.4.).

The generally progressive reduction in staining intensity of the protein bands at 11, 13, 24, 25, 27, 39-40, 43 and 46 kD on electrophoretic profiles of seminal plasma from the anterior ductus deferens to the ejaculate, and the disappearance of protein bands at 12.5, 15 and 22.5 kD from the ejaculated seminal plasma profile suggests selective reabsorption of proteins by the luminal epithelium of the ductus deferens and ampulla. Selective absorption of epididymal proteins by the avian ductus deferens has not previously been described. However, the occurrence of spermiphagy along the entire length of the male reproductive tract, and the selective and non-selective absorption of testicular fluid by the proximal efferent ductules have been reported (Tingari & Lake, 1972; Hess et al., 1982; Nakai et al., 1989).

Studies carried out in mammals have revealed similar spermiophagic and absorptive properties along the male reproductive tract (see Nakai et al., 1989).

Protein bands at 21, 49, 51, 66, and 80 kd exhibited an increase in staining intensity related to progression through the ductus deferens to the ejaculate (see 3.1.4.1.), indicating protein secretion throughout the ductus deferens. Esponda and Bedford (1985) also observed increased staining intensity of some seminal plasma proteins between the epididymis and posterior ductus deferens. The appearance of protein band complexes at 120 and 160 kd in ejaculated seminal plasma profiles may be due to secretion of new proteins and/or modification of existing seminal plasma proteins. Brooks and Tiver (1984) reported the accumulation of protein in rat epididymal fluid, due to the loss of testis specific antigens from the sperm surface. However, in the present study, some protein bands exhibiting a progressive increase in staining intensity between the anterior ductus deferens and ejaculate did not appear to associate with the sperm surface. The few that did, exhibited similar staining intensities on electrophoretic profiles of proteins removed following 1.0M NaCl treatment of spermatozoa recovered from the anterior and posterior ductus deferens (see 3.1 4.1.), suggesting that protein loss from the sperm surface related to progression through the ductus deferens was not generally occurring,: if protein loss

from the sperm surface occurred during posterior progression through the ductus deferens, then it would be expected that proteins in the electrophoretic profile of washings obtained from treatment of spermatozoa from the posterior ductus deferens with 1.0M NaCl would exhibit reduced staining intensity.

Around 25 seminal plasma proteins appeared to associate with the chicken sperm surface (see 3.1.4.1.). These were washed from the sperm surface using buffer containing 1.0M NaCl, and exhibited identical molecular weights to proteins in seminal plasma, thus indicating their seminal origin. These proteins also exhibited relative staining intensities on electrophoretic profiles which were in many cases considerably different from those observed for the same protein on seminal plasma profiles. This, and the observation that the intensely stained protein bands at 66 and 80 kd in seminal plasma profiles were absent from the electrophoretic profiles of sperm surface washings, indicates a selective uptake of proteins from seminal plasma by the sperm surface.

It has previously been demonstrated in mammals that some epididymal proteins are only weakly associated with the sperm surface and can be leached off by repeated washing or eluted with high ionic strength media (Rifkin & Olson, 1985; Brackett & Oliphant, 1975; Murphy & Carroll, 1987). However, others may insert into the plasma membrane or be anchored to the membrane by phosphatidylinositol and may

not be removed by perturbation of electrostatic forces (see Jones, 1989). Thus the possibility exists that some proteins or glycoproteins secreted by the chicken epididymis and/or ductus deferens and interacting with the sperm plasma membrane might not be extractable with 1.0M NaCl, and therefore not detected in this study.

The electrophoretic profiles of proteins obtained from anterior and posterior ductus deferens spermatozoa by 1.0M NaCl treatment, appeared very similar (see Figure 6), indicating that sperm surface-associated proteins extractable by hypertonic buffer treatment of spermatozoa are already associated with the sperm surface at the anterior ductus deferens. However, progressive changes in the antigenicities of seminal plasma proteins were observed between the anterior ductus deferens and the ejaculate (see Figure 7), indicating a role for the ductus deferens in sperm surface modification, possibly by conformational change of proteins already associated with the sperm surface and/or addition of secreted antigenic components. Furthermore, the glycosylation of the 80 kd seminal plasma protein in the ductus deferens, and several proteins in the ampulla suggests differential enzymic roles for the ductus deferens fluid in these regions.

In this study, slightly differing protein band staining intensities were observed on silver stained electrophoretic profiles of sperm surface washings obtained by hypertonic buffer treatment of ductus deferens

and ejaculated spermatozoa (see Figures 6, 15). It is clear that seminal plasma and sperm surface modification continues in the ampulla, and it is possible that this is accompanied by changes in the relative affinities of some proteins for the sperm surface. However, some variability is evident between experiments, in banding patterns of sperm surface-associated proteins extracted under similar conditions (see Figures 6, 9), although these may be partly explained by differences in experimental protocols used.

The absence of WGA-stained protein bands at 13, 13.5, 14 and 15 kD from posterior ductus deferens and ejaculated seminal plasma profiles and reduction in staining intensity of the band at 38 kD between the anterior ductus deferens and ejaculate (see 3.1.4.2.) may indicate deglycosylation in the ductus deferens, as has been reported in the mammalian epididymis (Brooks & Tiver, 1984). However, the lack of staining of bands at 13 and 15 kD from posterior ductus deferens and ejaculated profiles could be due to their progressive disappearance from seminal plasma.

Although protein bands on sperm surface washings profiles were of coincidental molecular weight to proteins appearing on seminal plasma profiles, they were generally absent from blotted profiles after immunological staining with antiserum raised against ejaculated seminal plasma proteins, suggesting antigenic modification as a result

of their removal from the sperm surface, probably due to conformational change.

Some sperm surface associated proteins appeared to undergo complex processing within the epididymis and ductus deferens. For example, the protein band at 11 kd appeared in all 3 silver stained seminal plasma profiles and was more intensely stained in the anterior ductus deferens profile. However, this protein expressed antigenicity only on posterior ductus deferens and ejaculated seminal plasma profiles, and was absent from all 3 WGA-stained seminal plasma profiles. It was, however, present on the WGA stained sperm surface washings profile from the anterior ductus deferens only, suggesting that it is secreted as a protein and subsequently glycosylated after association with the sperm surface. Lack of glycosylation of the free protein in seminal plasma might suggest a different conformation on the sperm surface. The glycoprotein appears to be subsequently deglycosylated in the ductus deferens. Toshimori et al. (1988) reported the masking of a mouse sperm surface-associated antigen with sialic acid in the distal caput epididymis, with subsequent desialylation in the cauda epididymis, resulting in antigenic expression. Toshimori et al. (1991) have suggested that sialylation might protect spermatozoa from spermophagy by macrophages and epithelial cells of the excurrent ducts. The lack of WGA staining of bands at 12.5, 22 and 23 kd in seminal plasma

profiles (see 3.1.4.2.) also suggests glycosylation of proteins after association with the sperm surface.

The presence of WGA-stained protein bands at 13, 13.5, 14, and 15 kD on the anterior ductus deferens seminal plasma profile may be indicative of glycoprotein secretion by the epididymal region, as has been reported for mammals. However, it is possible that glycosylation of secreted proteins may have occurred in the epididymal lumen, as observed in the ductus deferens and ampulla (see 3.1.4.2.).

Antisera raised against individual sperm surface-associated proteins were produced for use in charting the distributions of individual antigens on the sperm surface. However, although all antisera reacted with their homologous protein on Western blots of seminal plasma proteins separated by SDS-PAGE, they also cross-reacted with a number of other seminal plasma proteins, some of which also associated with the sperm surface (see 3.3.2.), and therefore were not specific enough for the detection of individual antigens. The cross-reactivity observed may indicate the presence of shared epitopes on different seminal plasma proteins. It is also possible that the use of SDS-treated proteins to raise antisera resulted in the production of antisera with altered specificities.

Protein bands stained by individual antisera after SDS-PAGE and Western blotting were clearly not merely

subunits of a single target protein, since some bands were stained by different antisera. Furthermore, the same 4 antisera, recently used to stain blotted seminal plasma proteins separated by native PAGE, each reacted with at least 3 high molecular weight (>250 kD) proteins (W. Meldrum & M.G. Steele, unpublished results).

The protein band at 11.5 kD in the electrophoretic profile of sperm surface washings from anterior ductus deferens spermatozoa was absent from the posterior ductus deferens profile, and that at 12.5 kD showed reduced staining intensity (see 3.1.4.1.), indicating the loss of these proteins from, or alteration of their affinities for, the sperm surface. The reduction in staining intensities of these proteins in sperm surface washings profiles was accompanied by a reduction in staining intensities in seminal plasma profiles, and may indicate their removal from the sperm surface, protein destruction in-situ by proteolysis, or internalisation of proteins by spermatozoa, with subsequent autocatalytic degradation (see Jones, 1989), accompanied by reabsorption from seminal plasma, by the luminal epithelium of the ductus deferens.

Esponda and Bedford (1985) separated chicken ductus deferens seminal plasma proteins by isoelectric focussing and observed only one band on the electrophoretic profile after staining with FITC-conjugated ConA. On the basis of that result, the report by Bedford (1979) that there

appeared to be no difference in the pattern of lectin staining of chicken spermatozoa recovered from the testis and ductus deferens, and the observation that there appeared to be little difference in the pattern of lectin staining of testis and ductus deferens spermatozoa from three related species of snake (Esponda & Bedford, 1987), led Esponda and Bedford (1987) to postulate that epididymal proteins rather than glycoproteins associate with the surface of subtherian vertebrate spermatozoa. This is clearly not the case for chicken ejaculated seminal plasma. A number of protein bands were stained with FITC-conjugated WGA, indicating that they are in fact glycoproteins. Furthermore, several sperm surface-associated glycoproteins are present in seminal plasma and/or sperm surface washings e.g. 13.5, 25 and 35 kd. Differences between results obtained in this study and that of Esponda and Bedford (1985) may have been due to the different lectins used, and different sources of seminal plasma.

It is clear that a great deal of glycosylation of proteins takes place in the ampulla of the ductus deferens (see 3.1.4.2.; figure 7). Moreover, proteins on blotting membranes are more accessible to high molecular weight probes than in gels (Stott, 1988), and chicken sperm surface moieties are considerably more intensely stained with WGA than with ConA (See 3.1.3. figures 4, 5). Thus incompletely glycosylated seminal plasma proteins reacting weakly with ConA may not have been visibly stained in the

earlier study. Alternatively, it is possible that the isoelectric points of chicken seminal plasma glycoproteins are similar, thus appearing as a single band after isoelectric focussing.

In an attempt to identify epididymal proteins which associate with the chicken sperm surface, Morris et al. (1987) reported the isolation of 4 such proteins at 13, 20 and 30-40 kD (2 bands). However, the immunoprecipitation technique used was dependent on protein antigenicity, and several of the sperm surface associated proteins identified in the present study, particularly those with low molecular weights, are either non-antigenic or exhibit very limited antigenicity (see 3.1.4.2.). Furthermore, in common with Esponda and Bedford (1985), Morris et al. (1987) used an antiserum raised against epididymal and ductus deferens seminal plasma proteins, which are generally less antigenic than their counterparts in ejaculated seminal plasma. Protein bands staining fairly intensely at 35 and 46 kD on sperm surface washings profiles could be the sperm surface associated proteins identified by Morris et al. (1987) at 30-40 kD, although the 35 kD protein appears only weakly antigenic (see 3.1.4.1., 3.1.4.2.). Similarly, those identified at 11 and 25 kD could be the other 2 proteins described by Morris et al. (1987) at 13 and 20 kD.

Most sperm surface-associated proteins exhibited similar staining intensities in electrophoretic profiles of sperm

surface washings obtained from the treatment of ejaculated spermatozoa with isotonic and subsequently hypertonic (1.0M NaCl) buffer (see 3.2.), indicating a range of affinities for the sperm surface, for individual proteins. This suggests that protein association with the sperm surface may be non-specific and possibly occurs via a number of different binding sites on the proteins and/or sperm surface. Proteins at 25, 38, 43, 87 and 124 kd were stained more intensely in the 1.0M NaCl sperm surface washings profiles (see 3.2.), indicating a stronger association with the sperm surface. Conversely, the proteins at 72 and 83 kd appeared to be very weakly bound.

Only seminal plasma components which could be found in the particulate fraction appeared to associate with the sperm surface (see 3.1.4.1., 3.2.). In many cases, these were found in considerably higher relative concentrations in the particulate fraction than in the soluble fraction. Furthermore, the similarities between the seminal plasma particulate fraction and 0.25M NaCl sperm surface washings profiles suggests that proteins which associate with the sperm surface may be derived from the particulate fraction of seminal plasma. If this is the case, then protein uptake from the particulate fraction may be selective, as differences were observed in the relative concentrations of some proteins in sperm surface washings and seminal plasma particulate fraction profiles (figure 8).

The particulate fraction of seminal plasma contains a high concentration of vesicles (G. J. Wishart, personal communication). Cells ultrastructurally suited to a secretory function are found throughout the male reproductive tract of domestic birds (Tingari, 1972; Hess et al., 1976; Bakst, 1980a). Low cuboidal cells, which are believed to secrete proteins (Tingari, 1972) are restricted to the rete testis. Non-ciliated type II cells, which probably also secrete proteins, are found mainly in the ductus epididymidis and ductus deferens, whereas non-ciliated type I cells, which are believed to secrete non-proteinaceous material, are restricted to the ductuli efferentes and connecting ductules (Tingari, 1972). Cells of the ductuli efferentes (Bakst, 1980a; Hess et al., 1976) and ductus epididymidis and ductus deferens (Tingari, 1971; Hess et al., 1976) exhibit 'blebbing' into the ductule lumen, which may be indicative of apocrine secretion (Tingari, 1971; Hess et al., 1976), unlike the holocrine secretion reported for mammals (see Tingari, 1971). Furthermore, Hess et al. (1982) observed membrane-bound electron dense cytoplasmic bodies forming cytoplasmic blebs which extended into the lumen of the ductuli efferentes. Thus it would appear that epididymal proteins may be secreted in vesicles and that proteins associated with vesicles subsequently associate with the sperm surface. It is possible that such vesicles disintegrate within the lumen of the excurrent duct system, releasing their contents, which subsequently

associate with the sperm surface. Certainly, most of the protein of seminal plasma is in the soluble fraction.

4. 1. 2. The Sperm Surface

Chicken spermatozoa exhibited clear changes in the pattern and intensity of staining with WGA and lectin from Solanum tuberosum during transport through the excurrent duct system (see 3. 1. 3.).

Changes in lectin binding affinity occurred in the epididymal region between the testis and anterior ductus deferens, and comprised a general increase in staining intensity and uniformity for WGA and the appearance of carbohydrate groups recognised by lectin from Solanum tuberosum.

Lectins are a distinctive group of polyvalent proteins which bind to carbohydrate residues of specific structure and configuration (Goldstein & Hayes, 1978). They are classified according to their recognition of monosaccharides located at the non-reducing end of carbohydrate chains (class I) e.g. ConA, lectin from Arachis hypogaea; or carbohydrate sequences (class II) e.g. WGA, lectin from Solanum tuberosum (Gallagher, 1984). WGA recognises a number of different oligosaccharide groups and sialic acids (see Appendix 1 for lectin specificities), including N^I, N^{II}, N^{III}-triacylchitotriose [(GlcNAcB1-4)₃]. Lectin from Solanum tuberosum also recognises the sequence specifically and confirmed its appearance on the sperm surface during transport through the epididymal region of

the excurrent duct system. The agglutination and intense, uniform staining observed on ejaculated spermatozoa after exposure to lectin from Limulus polyphemus (see 3.4.3.1.) indicates the predominance of sialic acid residues on the chicken sperm surface. Although no overall difference in the intensity or uniformity of WGA staining was apparent between ejaculated spermatozoa and spermatozoa recovered from the anterior ductus deferens, it is possible that subtle changes may have occurred, but were not detected against the high staining intensity.

Affinity of spermatozoa for ConA remained unchanged between the testis and ejaculate, indicating the acquisition of α -D-glucose, α -D-mannose and/or N-acetyl-D-glucosamine residues during sperm transport through the excurrent duct system, in a similar distribution to those existing on the testicular sperm surface. In contrast to the distribution of binding sites for WGA and lectin from Solanum tuberosum along the whole sperm length, carbohydrate residues recognised by ConA exhibited regional distribution. Bakst and Howarth (1977a) reported staining over the entire length of chicken spermatozoa after exposure to HRP conjugated ConA. However, spermatozoa were examined at X79000 magnification using electron microscopy, thus allowing a considerably greater degree of resolution than was possible in this study.

Lectins from Arachis hypogea and Erythrina corralloendron did not react with the surface of spermatozoa from the ejaculate, ductus deferens or testis, indicating a lack of β -D-Gal-(1-3)-D-GalNAc and β -D-Gal-(1-4)-D-GlcNAc residues on the chicken sperm surface.

The increased binding of WGA and lectin from Solanum tuberosum to the sperm surface during transport through the epididymal region may be due in part to glycosylation of existing plasma membrane components, given the continued glycosylation of proteins throughout the ductus deferens and in the ampulla (4.1.1.). In mammals, galactosyltransferase, sialyltransferase and α -lactalbumin-like proteins have been detected in epididymal secretions and evidence has been obtained for the glycosylation of existing membrane components (see Jones, 1989). Although deglycosylation of existing membrane components appears to occur in the ductus deferens (see 3.1.4.2.), no apparent reduction in sperm staining intensity was observed. This may have been due to a low concentration of deglycosylated components on the sperm surface and the polyvalent nature of WGA, possibly allowing cross-linking over the deglycosylated proteins.

Another possible explanation for the increased lectin binding observed accompanying sperm transport through the epididymal region is the association of secreted glycoproteins with the sperm surface. In mammals it is now widely accepted that glycoproteins synthesised and

secreted by the epididymal epithelium associate with the sperm surface (Olson & Hamilton, 1978; Jones et al., 1981; Moore, 1980; Orgebin-Crist & Fournier-Delpech, 1982; Brooks, 1983).

Bedford (1979) reported no change in WGA or ConA binding to the chicken sperm surface as a correlate of sperm transport from the testis to the ductus deferens, and postulated that, in contrast to the case in mammals, cell surface change might not be a feature of post-testicular maturation in the chicken. Conflicting results obtained by Nicolson et al. (1977) and Gordon et al. (1975) regarding the binding of lectins to rabbit spermatozoa during epididymal transit have been attributed to differences in experimental protocols (see Olson, 1984). It is possible that differences between results obtained in this study and those reported by Bedford (1979) for WGA binding to the sperm surface were also due to differences in staining protocols.

Esponda and Bedford (1987) reported no changes in ConA or WGA binding over the surfaces of snake, turtle or lizard spermatozoa correlated with sperm transport from the testis to the anterior ductus deferens, and, as previously mentioned (see 4.1.1.), subsequently suggested that sperm surface modification in subtherian vertebrates might involve the acquisition of proteins rather than glycoproteins. However, given that epididymal antigens bind over the entire sperm surface (see 3.1.2.), it would

be reasonable to expect that the acquisition of protein rather than glycoprotein would mask underlying lectin binding sites of testicular origin and result in reduced lectin binding correlated with sperm transport through the excurrent duct system. This is clearly not the case in the chicken.

No staining differences were observed between fixed and unfixed spermatozoa following exposure to FITC conjugated lectins (see 3.1.3.), suggesting that the fixative used (formaldehyde) did not expose intracellular components. However, spermatozoa recovered from the anterior oviduct following multiple intravaginal inseminations exhibited more extensive staining after fixation (see 3.6.1.), suggesting intracellular staining or unmasking of surface groups. It has previously been shown that some fixatives can expose intracellular components often giving rise to increased staining of fixed spermatozoa with some lectins, compared with unfixed spermatozoa (see Cross & Overstreet, 1987), and it is possible that a small increase in the staining intensity of fixed spermatozoa due to WGA labelling of internal components was not detected against the intense sperm surface staining. It would also seem likely from the results obtained that the sperm interior lacks significant binding sites for ConA and lectin from Solanum tuberosum.

Esponda and Bedford (1985, 1987) have shown clearly that the antigenicity of the sperm surfaces of chicken and some

species of snake undergo dramatic change during sperm transport through the excurrent duct system. The immunofluorescence results obtained in this study from the exposure of ejaculated spermatozoa and spermatozoa recovered from different regions of the male reproductive tract, to antiserum raised in rabbit against ejaculated seminal plasma proteins, were broadly in agreement with those obtained by Esponda and Bedford (1985) (see 3.1.2.). Testicular spermatozoa generally remained unstained, with all spermatozoa examined from the ductus deferens and ejaculate exhibiting staining.

Esponda and Bedford (1985) examined spermatozoa from the epididymal region, mid-ductus deferens and posterior ductus deferens and observed incomplete staining of epididymal spermatozoa, with complete staining of spermatozoa from the ductus deferens. In this study, spermatozoa recovered from the anterior ductus deferens exhibited staining over their entire surface, similar to that observed on ejaculated spermatozoa and spermatozoa recovered from the posterior ductus deferens. However, staining intensity increased progressively from the anterior ductus deferens to the ejaculate, indicating progressive change in sperm surface antigenicity, and was accompanied by changes in the antigenicities of seminal plasma proteins (see 3.1.4.2.). This is an important difference to the results obtained by Esponda and Bedford (1985), as the results from this study suggest that the entire ductus deferens is actively involved in

post-testicular sperm maturation, supporting the view that the ductus deferens is the avian equivalent of the corpus and caudal regions of the mammalian epididymis (Tingari, 1971, 1972; Lake, 1981). It is possible that sperm surface staining differences observed between this study and that of Esponda and Bedford (1985) were due to the different origins of the antisera used. Esponda and Bedford (1985) raised antisera against seminal plasma from the epididymis and ductus deferens, whereas in this study ejaculated seminal plasma was used. As previously mentioned (4.1.1.), it is clear that differences exist between the antigenicities of ejaculated seminal plasma proteins and their counterparts from different areas of the ductus deferens.

A small percentage of ejaculated and ductus deferens spermatozoa from all birds used exhibited considerably more intense immunological staining of the head region than that generally observed (see 3.1.2.). Similarly, a larger percentage of ejaculated and ductus deferens spermatozoa exhibited incomplete staining after exposure to FITC conjugated WGA (see 3.1.3.). These observations appeared fairly constant in sperm samples examined from different birds, indicating the existence of sperm subpopulations within semen samples.

Sperm heterogeneity within semen samples has been demonstrated in several mammalian species (see Holt, 1984), using murine monoclonal antibodies (Glassy et al.,

1984), continuous percoll gradient centrifugation (Sullivan & Robitaille, 1989), aqueous two-phase partition (Cartwright et al., 1992), lectins (Ravid et al., 1990) and anti-sperm antibodies (Lenzi et al., 1988). Glassy et al. (1984) postulated that cross-mixing of surface antigens may occur between cells throughout sequential rounds of cell division during spermatogenesis, and that the persistence of maturation antigens on mature spermatozoa may give rise to antigenic heterogeneity. Sullivan and Robitaille (1989) suggested that heterogeneity of sperm populations may be related to varying degrees of maturation of spermatozoa within any given population. It would appear that the distribution of antigens on the sperm surface may be an important factor in the fertilising ability of spermatozoa. Blaquier et al. (1987) reported a study in which 42% of infertile men exhibited abnormal distribution of sperm surface antigens.

A small percentage of testicular spermatozoa from each bird reacted with the antiserum raised against seminal plasma proteins (see 3.1.2.), indicating heterogeneity of the sperm surface prior to the acquisition of epididymal antigens. It is possible that some components on the surface of a small percentage of testicular spermatozoa possess similar epitopes to antigens present in seminal plasma. Alternatively, one or more antigens present in seminal plasma which associate with the sperm surface might originate in the testes. Orlando et al. (1988) have

reported an albumin secreted by mammalian Sertoli cells which is also present in seminal plasma, and Bardin et al. (1986) reported the secretion of a protein by Sertoli cells and the epididymal epithelium.

4. 2. Oviducal Sperm Selection

4. 2. 1. The Uterovaginal Junction as a Site of Sperm Selection

The hypothesis that the uterovaginal junction sperm storage tubules of the chicken hen are selective with regard to the spermatozoa that enter them (Allen & Bobr, 1955; Allen & Grigg, 1957; Ogasawara et al., 1966) has been disproved by evidence provided in this thesis. In addition to spermatozoa from various avian species, spermatozoa from several mammalian species were observed to enter quail uterovaginal junction SSTs in-vitro, in some cases filling the tubule lumen and adopting a similar storage orientation to the host avian spermatozoa (see 3.5.1.). Furthermore, human spermatozoa occupied a small percentage of quail uterovaginal junction SSTs following intravaginal insemination, and subsequently gained access to the newly ovulated egg over 4 consecutive days (see 3.5.3.3.), indicating longer-term storage. The decline in the concentration of spermatozoa observed in the perivitelline layer of subsequently laid eggs appeared logarithmic, as is believed to be the case for homologous avian spermatozoa (Wishart, 1987), suggesting that human spermatozoa were retained in the SSTs and subsequently released in a similar fashion to homologous avian spermatozoa.

It would seem fair to assume that human spermatozoa observed in the perivitelline layer of quail eggs would have been released from the uterovaginal junction SSTs rather than infundibular SSTs. Although sperm transport from the uterovaginal junction to the infundibulum is believed to be passive (Fujii & Tamura, 1963; Van Krey et al., 1967), it is likely that spermatozoa require to be motile in order to enter SSTs.

In this study, chicken spermatozoa often failed to reach the infundibulum or were found there only in very small numbers 3-4 hours following uterovaginal junction insemination into chicken hens.

The small dimension of the quail vagina precluded efficient intravaginal insemination in this study, with the result that some inseminated spermatozoa were likely to have been introduced directly into the posterior uterovaginal junction. Efficient interspecies sperm selection in the vagina would suggest that spermatozoa observed in uterovaginal junction SSTs were inseminated into the uterovaginal junction. However, given the alien environment of the avian oviduct (which has a temperature 3-4 °C above normal mammalian body temperature), it would seem unlikely that human spermatozoa would have remained motile long enough to enter infundibular SSTs in sufficient numbers for subsequent detection in eggs released over 4 consecutive days. Furthermore, in an earlier report, heterologous spermatozoa from 4 mammalian

species suffered a considerable decrease in motility 1 hour following intrauterine insemination into hamsters (Smith et al., 1988).

The appearance of spermatozoa on the perivitelline layer of 4 consecutive eggs from the same hen would have necessitated sperm storage as the infundibular or magal egg fills the entire oviducal lumen, thus flushing luminal spermatozoa through the oviduct. Although spermatozoa stored at the uterovaginal junction SSTs did not appear to have been maintained in a viable state within the tubules (see 3.5.3.3.), passive transport of spermatozoa from the uterovaginal junction to the infundibulum would have allowed even dead spermatozoa to appear on the perivitelline layer of subsequently ovulated eggs.

Lake and Ravie (1988), upon observing reduced chicken sperm fertilising ability following removal of sperm surface N-acetylneuraminic acid residues with neuraminidase, suggested that sperm selection might be based on a recognition system between the sperm storage tubule and the sperm surface. However, Schindler et al. (1967) reported that spermatozoa generally lie free within the lumen of the SSTs and are not in direct contact with the luminal epithelium. Furthermore, in this study, antisera raised against chicken and quail seminal plasma proteins did not cross-react with the human or bovine sperm surface (see 3.5.2.), indicating the probable lack of any such recognition protein on the mammalian sperm

surface. It would certainly seem unlikely that mammalian spermatozoa would possess components on their surface designed for recognition within an avian sperm storage system. Moreover, chicken spermatozoa treated with either neuraminidase or buffer containing 0.25M NaCl, which exhibited reduced access to homologous uterovaginal junction SSTs in-vivo (see 3.4.3.3., 3.4.1.3. respectively) gained comparable access to the SSTs in-vitro to that of their respective controls (see 3.5.3.2.), indicating that the uterovaginal junction SSTs are not selective for spermatozoa.

Ogasawara et al. (1966) suggested that the uterovaginal junction SSTs might select spermatozoa on the basis of their morphology. However, although spermatozoa from different avian species incubated in-vitro with uterovaginal junction tissue were of similar morphology, the mammalian spermatozoa used were variable in length and head morphology, which invariably differed greatly from avian spermatozoa. Furthermore, chicken spermatozoa rendered morphologically abnormal after treatment with 0.4M NaCl were commonly found in quail uterovaginal junction SSTs after in-vitro incubation (see 3.5.1.).

4.2.2. The Role of Sperm Surface Associated Proteins in the Oviduct

Proteins and glycoproteins can clearly be extracted from the surface of ejaculated chicken spermatozoa using isotonic and hypertonic buffers containing up to 0.25M NaCl, without apparent loss of sperm integrity as determined by assay of cellular ATP concentration, motility and eosin exclusion. However, spermatozoa treated with 0.25M NaCl exhibited significantly reduced access to the uterovaginal junction SSTs in-vivo, and hypertonic buffer-treated spermatozoa showed a significant NaCl concentration-related reduction in ability to reach the site of fertilisation following intravaginal insemination (see 3.4.1.1., 3.4.1.3.), indicating a definite role for sperm surface associated proteins in vaginal sperm transport.

The principle of removing sperm surface-associated proteins of epididymal origin with isotonic or high ionic strength media has previously been demonstrated for mouse (Murphy & Carroll, 1987; Rankin et al., 1992a), rabbit (Brackett & Oliphant, 1975; Oliphant & Singhas, 1979); rat (Rifkin & Olson, 1985), boar (Russell et al., 1985) and lizard (Depeiges & Dafaure, 1983). However, in those experiments it was not intended to retain viable spermatozoa, unlike the present study in which the metabolic and functional intactness of the spermatozoa were retained in-vitro during and after washing, both to

define the topical source of the proteins and to provide a system whereby the effect of protein removal on sperm function in-vivo could be determined.

The idea that avian sperm surface-associated proteins may confer upon spermatozoa the ability to traverse the female reproductive tract has been suggested previously (Esponda & Bedford, 1985; Morris & Howarth, 1985). However, the evidence, that testicular spermatozoa which have not been exposed to the secretions of the epididymis are infertile when inseminated intravaginally (Munro, 1938b; Howarth, 1983), but fertile when inseminated intramagnally (Howarth, 1983), could be considered equivocal, since testicular spermatozoa also display limited motility in-vitro (Munro, 1938b; Howarth, 1983; Ashizawa & Sano, 1990).

It is clear that sperm surface-associated proteins do not have a role in sperm-egg interaction (Howarth, 1983) or the development of motility (see Bedford, 1979) in the chicken, although acrosome stabilisation (Eng & Oliphant, 1978) remains a possibility. However, the results obtained in this study point to a role in sperm transport in the posterior oviduct.

Reincubation of spermatozoa with buffered seminal plasma following exposure of spermatozoa to 0.25M NaCl failed to improve the ability of spermatozoa to reach the site of fertilisation. This could simply have been due to

inappropriate timescale of incubation. However, given the degree of modification of epididymal proteins in subsequent areas of the excurrent duct system, it is possible that proteins of ejaculated seminal plasma are in an inappropriate form for association with the sperm surface. In this study, proteins extracted from the sperm surface by hypertonic buffers were not recognised by the antiserum raised against seminal plasma proteins (see 3.1.4.2.), suggesting antigenic modification, probably as a result of conformational change. Thus it may not be unreasonable to expect that sites on the sperm surface for protein association could also have been altered, preventing protein-sperm surface reassociation.

Sperm surface-associated proteins and glycoproteins are clearly lost from spermatozoa washed with isotonic buffer (see 3.4.1.2.). Simple centrifugation with subsequent reconstitution of the pellet in the same buffered seminal plasma results in a significant reduction in sperm access to the newly ovulated egg in-vivo, a situation which is clearly exacerbated by a change of buffer (see 3.4.2.2.). Given that cellular damage due to centrifugation, reconstitution of the pellet and withdrawal of seminal plasma was not evident during a prolonged 80 minute aerobic incubation at 40 °C (see 3.4.2.1.), it would seem reasonable to assume that detrimental effects on the ability of spermatozoa to reach the site of fertilisation were largely due to loss of sperm surface associated proteins and glycoproteins. It may be possible for

proteins removed from the sperm surface in isotonic conditions to reassociate with spermatozoa during subsequent sperm incubation in the presence of seminal plasma, as may have occurred for spermatozoa which had been centrifuged only, thus allowing a degree of recovery of normal sperm surface presentation. This could account for the considerably greater ability of such spermatozoa to reach the site of fertilisation than their washed counterparts, and their relatively poor performance compared with uncentrifuged spermatozoa. Certainly, the acquisition of epididymal antigens by immature mammalian spermatozoa in-vitro has been reported (see Moore, 1990). It is also possible that removal of seminal plasma prior to insemination renders spermatozoa more vulnerable to immunological attack in the oviduct. Immunosuppressive factors similar to those identified in mammalian semen (see Alexander & Anderson, 1987; Metafora et al., 1989) may be present in chicken seminal plasma.

In practice, the loss of sperm surface-associated proteins may be a cause of lowered fertility in hens inseminated with treated chicken semen. Simple dilution of chicken semen at 10- and 46-fold in a buffered glutamate-based diluent resulted in, respectively, 19 and 34% reductions in fertility, in terms of the percentage of fertile eggs laid by inseminated hens, compared to samples similarly diluted in chicken seminal plasma (Lake & Ravie, 1987). The problem may be more acute during protocols for cryopreservation of spermatozoa, utilising glycerol as the

cryopreservative, when semen samples are not only diluted in a medium of high osmolarity, but are also washed free of glycerol and seminal plasma by centrifugation prior to insemination (e.g. Lake et al., 1981)

The removal of terminal N-acetylneuraminic acid residues from the sperm surface without adversely affecting the metabolic and functional intactness of spermatozoa, as assessed by measurement of cellular ATP concentration, motility and eosin exclusion (see 3.4.3.2.), resulted in reduced access of spermatozoa to the uterovaginal junction SSTs following intravaginal insemination, when compared with untreated controls (see 3.4.3.3.). Thus terminal sialic acid residues on the sperm surface are also important in vaginal sperm transport and/or storage in chickens. Previous work has shown that up to 80% of chicken seminal sialic acid content is associated with spermatozoa (Froman & Engel, 1989) and that the removal of 18-45% of sperm bound sialic acid results in reduced sperm fertilising ability (Froman & Thurston, 1984; Lake & Ravie, 1988; Froman & Engel, 1989; Howarth, 1989).

Van Krey et al. (1981) suggested that storage of spermatozoa within the uterovaginal junction SSTs depends on their ability to agglutinate. Froman and Thurston (1984), Froman and Engel (1989), and Howarth (1989) subsequently postulated that the reduced fertilising ability of neuraminidase treated spermatozoa is due to loss of sialic acid residues responsible for sperm

agglutination and storage in the SSTs. In this study, intravaginally inseminated spermatozoa subsequently recovered from the uterovaginal junction or infundibulum exhibited reduced staining with FITC-conjugated WGA (see 3.6.1.). However, spermatozoa recovered from the vagina up to 5 hours following intravaginal insemination stained similarly to non-inseminated control spermatozoa, suggesting that loss of sperm surface carbohydrate residues (probably terminal sialic acid) occurs naturally at the uterovaginal junction, and lending support to the sperm agglutination hypothesis. However, neuraminidase treated spermatozoa were not generally found in the uterovaginal junction SSTs or on the uterovaginal junction mucosa 3 hours following intravaginal insemination (see 3.4.3.3.), at which time filling of SSTs with spermatozoa would normally be proceeding (Allen & Grigg, 1957; Bobr et al., 1964b). This strongly suggests that rather than not being retained by the SSTs, inseminated spermatozoa were prevented from reaching the uterovaginal junction by some mechanism operating in the vagina.

Probing of the chicken sperm surface with FITC-conjugated lectin from Limulus polyphemus indicated a uniform distribution of sialic acid residues over the entire surface (see 3.4.3.1.). Sialic acids bound to cell surfaces are believed to carry out a wide range of functions, including the aggregation of cells via Ca^{2+} bridges, and the masking of antigenic sites (see Schauer, 1985). Kelm et al. (1986) reported that erythrocyte

binding to liver, spleen and peritoneal macrophages, and subsequent phagocytosis was due to loss of cell-surface sialic acid. Sialic acids are also known to decrease the reactivities of IgG with Fc receptors on T lymphocytes, and with Killer T cells (see Schauer, 1985). The masking mechanism of sialic acid is presently unknown, but may be due to influence over glycoprotein conformation or a simpler steric effect (Schauer, 1985; Toshimori et al., 1991).

4.2.3. Glycerol and Avian Spermatozoa

Glycerol is the most extensively used cryoprotective agent for chicken spermatozoa (see Lake, 1986). A glycerol concentration of more than 7% is required for the most effective survival of chicken spermatozoa after freezing (Watanabe, 1967; Watanabe & Terada, 1980). However, although chicken spermatozoa have been shown to be highly active in-vitro in media containing up to 20% glycerol (v/v) at room temperature, unless the glycerol concentration is reduced to less than 2% (v/v) by dialysis or centrifugation prior to intravaginal insemination, spermatozoa subsequently exhibit greatly impaired fertilising ability (Polge, 1951). This appears to be due to impaired sperm migration from the vagina to the uterovaginal junction SSTs (Westfall & Howarth, 1977), although spermatozoa inseminated intrauterinely in a

medium containing 15% glycerol (v/v) subsequently fertilised eggs (Allen & Bobr, 1955).

Glycerol removal from spermatozoa must be carried out slowly to prevent osmotic damage (Bakst & Howarth, 1977a; Westfall & Howarth, 1978), due to dilution of glycerol-permeated spermatozoa with a glycerol-free medium.

Hammerstedt and Graham (1991) have postulated that glycerol might affect cytoplasmic viscosity and subsequently the response time of diffusion-limited processes. Direct alteration of membrane bilayer structure by glycerol has been reported and alterations to the sperm surface have been postulated (see Hammerstedt and Graham, 1991).

It is believed that the contraceptive effect of glycerol in the chicken vagina may be due to damage to the sperm plasma membrane as a result of rapid extraction of glycerol (Allen & Bobr, 1955; Westfall & Howarth, 1977).

The results obtained from this study indicate that several sperm surface-associated proteins are lost at room temperature from chicken spermatozoa as a result of exposure to buffer containing 5% glycerol (a concentration lower than that used in the cryopreservation of spermatozoa), which are not lost from control spermatozoa (see 3.4.4.2.), confirming the postulation that glycerol

modifies the sperm surface (Hammerstedt and Graham, 1991). Furthermore, proteins with molecular weights of 21 and 25 kd appear to be removed in a glycerol concentration-dependent manner.

However, the presence of glycerol appears to have prevented the extraction of the 63 kd protein from the sperm surface. The protection of cell surface proteins by glycerol has previously been reported by Ballas (1981), who showed that the presence of glycerol prevented elution of glyceraldehyde-3-phosphate dehydrogenase from the surface of erythrocytes by hypertonic buffers.

Weaker protein band staining on electrophoretic profiles of sperm surface washings obtained from exposure of spermatozoa to glycerol at 5 °C indicates a temperature-dependency for the protein extracting activity of glycerol, although extraction of proteins by isotonic buffer appears relatively temperature-independent.

Sperm integrity was retained following glycerol removal at room temperature, as assessed by measurement of cellular ATP concentration, sperm morphology and eosin exclusion, but motility was reduced in a glycerol concentration related manner (see 3.4.4.1.). Sperm integrity was also maintained after exposure to glycerol at 5 °C, with only spermatozoa exposed to 8.0% glycerol exhibiting a significant reduction in motility (see 3.4.4.1.). Such results would appear to contradict findings from previous

studies. Bakst and Howarth (1977a) and Marquez and Ogasawara (1977) reported plasma membrane disruption after incubation of chicken and turkey spermatozoa respectively, with media containing glycerol. However, in both cases, considerably higher glycerol concentrations (15% for chicken, 12.5% for turkey) were used than in this study. Furthermore, the experimental protocol described by Marquez and Ogasawara (1977) did not include the duration of exposure to glycerol or the temperature at which the incubation was carried out. However, both studies utilised electron microscopic examination of spermatozoa at high magnifications, and it is thus possible that subtle plasma membrane damage was not identified by light microscopic examination in this study. Westfall and Howarth (1978) reported the release of glutamic oxaloacetic transaminase, as an indicator of plasma membrane damage, after removal of glycerol from chicken spermatozoa. However, spermatozoa were held in glycerol containing media for 2 hours prior to centrifugation, compared with the 15 minute incubation used in this study.

The general reduction in sperm surface-associated protein removal by glycerol at 5 °C and the corresponding maintenance of sperm motility might suggest a direct relationship between reduction in motility and sperm surface associated protein removal. However, the lack of protein removal by 0.5 and 2.0% glycerol at room temperature would appear to contradict this hypothesis, given that sperm motility was reduced at these

concentrations, although an indirect relationship via disruption of plasma membrane cation transport is possible. Calcium stimulates chicken sperm motility when present at an extracellular concentration of 2 mM or greater (Wishart & Ashizawa, 1987). Westfall and Howarth (1977) reported significantly lower calcium and magnesium concentrations in unfrozen chicken spermatozoa following the removal of 15% glycerol by centrifugation than in non-glycerolised spermatozoa, suggesting a disturbance of calcium transport across the plasma membrane which could possibly account for the apparent glycerol concentration related reduction in sperm motility observed, possibly via the alteration of plasma membrane pore structures (see Hammerstedt & Graham, 1991).

Bakst and Howarth (1977a), using electron microscopy, reported no difference in the binding patterns of HRP conjugated ConA or cationised ferritin to control chicken spermatozoa or spermatozoa exposed to 15% glycerol, and subsequently concluded that loss of fertility following intravaginal insemination of glycerol treated spermatozoa was not associated with alteration of sperm surface glycoproteins which bind ConA and cationised ferritin. However, it is clear that the removal of sperm surface associated proteins, including glycoproteins at 25 and 53 kD (see 3.4.4.2., 3.1.4.1., 3.1.4.2.) is elicited by glycerol at concentrations considerably lower than 15%. A possible explanation for results obtained by Bakst and Howarth (1977a) might be derived from the observation made

in this study that ConA binding to the sperm surface appears unaffected by post-testicular maturation (see 3.1.3.). Thus removal of sperm surface-associated glycoprotein is likely to expose similar underlying ConA binding groups of testicular origin. This may also be the case for anionic sites recognised by cationised ferritin.

Given the clear importance of sperm surface associated proteins to sperm function within the oviduct and the requirement for motility in order to traverse the vagina (Takeda, 1974), it is possible that sperm surface protein removal and reduction in motility may be involved in the contraceptive effect of glycerol in the chicken vagina.

4.2.4. Assays of Sperm Viability

The use of motility, cellular ATP and eosin exclusion to assess sperm viability following treatment with hypertonic buffers, neuraminidase or glycerol would appear valid, since these functions require an intact axoneme and plasma membrane, and coupled mitochondria. In normal ejaculates from individual birds these functions are highly correlated with sperm fertilising ability (Wishart & Palmer, 1985). However, with cryopreserved chicken spermatozoa they greatly overestimate fertilising ability, and integral damage to spermatozoa is only revealed after prolonged incubation in-vitro (Wishart & Palmer, 1986).

In this study, the poor performance of washed and hypertonic buffer-treated (0.20 and 0.25M NaCl) spermatozoa in-vivo, and the reduced motility of glycerol treated spermatozoa in-vitro could not be shown to be the result of latent damage to spermatozoa, which maintained normal ATP concentrations in-vitro during prolonged incubation at 40 °C.

Spermatozoa treated with neuraminidase exhibited a significant reduction in ATP concentration between 20 and 80 minutes incubation at 40 °C. However, a corresponding reduction in control sperm ATP concentrations would suggest that increased handling of spermatozoa compared with that during hypertonic buffer or glycerol treatment protocols may have been a major causitive factor.

4.2.5. Site of Oviducal Sperm Selection in the Chicken

Turkey and chicken spermatozoa exhibit similar morphological (Lake & Wishart, 1984) and motility (Wishart & Ross, 1985) characteristics. Morris et al. (1987) reported that turkey spermatozoa exposed to an antiserum raised against chicken ductus deferens seminal plasma proteins were stained similarly to homologous chicken spermatozoa, suggesting shared sperm surface antigenicity. However, the finding in this study that an antiserum raised against chicken ejaculated seminal plasma proteins continued to bind extensively to the chicken sperm surface

following exhaustive absorption with turkey spermatozoa (see 3.5.3.1.), indicates that chicken spermatozoa have surface epitopes distinct from those on turkey spermatozoa.

Heterologous inseminations of turkey spermatozoa into chicken hens were carried out to produce an exaggerated challenge to the sperm selection mechanism present in the posterior region of the female reproductive tract. The results obtained (see 3.5.3.3.), and those obtained from the incubation of turkey spermatozoa with chicken SSTs in-vitro (see 3.5.3.2.), demonstrate that on the basis of a comparison of their access to chicken and turkey spermatozoa, the uterovaginal junction SSTs of the chicken hen do not exhibit any selectivity for their homologous spermatozoa either in-vitro or in-vivo. However, selection against heterologous turkey spermatozoa does appear to take place in the vagina, which permits migration of chicken but not turkey spermatozoa.

These findings are supported by previous experiments carried out by Takeda (1974) who observed that bovine spermatozoa did not migrate through the vagina of chicken hens, and Nash et al. (1986) who reported entry of bovine spermatozoa to chicken uterovaginal junction SSTs in-vitro.

The existence of a vaginal sperm selecting mechanism invokes the question of the basis upon which spermatozoa

are selected. Takeda (1974) considered that spermatozoa were selected for transvaginal migration on the basis of their motility, since dead (immotile) spermatozoa did not pass through the vagina. In this study, turkey spermatozoa appeared vigorously motile in-vitro and were clearly sufficiently active to enter chicken uterovaginal junction SSTs in-vitro and in-vivo (see 3.5.3.2., 3.5.3.3.).

Whilst it is not possible to ascertain the motility of turkey spermatozoa in the chicken vagina in-vivo, it seems unlikely that conditions of ionic milieu, pH, substrate, or oxygen tension within the vagina would affect spermatozoa of the 2 species differently. Although chicken and turkey spermatozoa differ in their energy metabolism in that turkey, but not chicken spermatozoa, have an obligatory requirement for oxygen to maintain optimal ATP concentrations (Wishart, 1982), the oxygen tension of the chicken and turkey vagina as determined in this study ($PO_2 = 58 \pm 1$ for turkey and 51 ± 7 for chicken) (Steele & Wishart, 1992) would be sufficient to support oxidative metabolism of spermatozoa from both species. The spermatozoa also have their own internal oxidative substrate (Wishart, 1982) and so would seem unlikely to require this from the vagina. Furthermore, whilst chicken but not turkey (Wishart, 1984a) spermatozoa become immotile in-vitro in simple buffers at the avian body temperature of 40-41 °C (Ashizawa & Wishart, 1987), the calcium content of most body fluids, and certainly

that extruded from the vagina at oviposition, would be sufficient to maintain chicken sperm motility at 40 °C (Wishart & Ashizawa, 1987). The pH of the chicken and turkey vagina are also similar (Bakst, 1980b).

4.2.6. Physiological Basis of Vaginal Sperm Selection in the Chicken

It is clear that chicken immunoglobulin binds to the surface of a large proportion of intravaginally inseminated homologous or heterologous spermatozoa within the initial 20 minute period following insemination, that a slightly smaller proportion are dead, and that these proportions increase with elapsed time following insemination (see 3.5.4.1.). Vaginal lavage was carried out initially 20 minutes following insemination, in light of a report by Howarth (1971), that more than 80% of intravaginally inseminated spermatozoa are mechanically ejected from the chicken vagina within 15 minutes of insemination with homologous spermatozoa. Thus the contents of the vagina during the initial 15 minute period following insemination are likely to be in a considerably greater state of flux than at times following this period, making assessment of sperm selection mechanisms difficult to study.

Kimijima et al. (1990) reported roughly equivalent numbers of IgG- and IgA-containing cells in the chicken vaginal

mucosa. The rabbit antichickens IgG used in this study cross-reacts with IgA (Sigma Chemicals, personal communication) and consequently recognises both isotypes. In mammals, both IgG and IgA are present in the secretions of the female reproductive tract (Beer & Neaves, 1978; Menge & Behrman, 1980; Cropp & Schlaaff, 1990). Although IgG has been implicated as the major sperm-binding isotype in the oviduct (Austin, 1960; Cohen & Werrett, 1975; Allen & Bourne, 1978; Taylor, 1982b), IgA can bind antigens efficiently and can activate complement via the alternative pathway (see Underdown & Schiff, 1986). Thus it is possible that either or both isotypes were present on spermatozoa stained with antichickens IgG.

Austin (1960), Symons (1967), Cohen and Werrett (1975) and Cohen and Tyler (1980) identified presumed subpopulations of mammalian spermatozoa by the presence or absence of IgG bound to the acrosome by the Fc region (Cohen, 1978; Allen & Bourne, 1978). However, Taylor (1982a) and Hancock (1984) suggested that lack of IgG binding to 'selected' spermatozoa may be due to loss of acrosomes. In this study however, most immunoglobulin binding occurred over areas of the flagellum, with little recognisable acrosomal binding, and therefore cannot be rationalised in the same manner.

Chicken immunoglobulin on the surface of spermatozoa recovered from the chicken vagina was detected in this study by indirect immunofluorescence of spermatozoa in

suspension. Previous use of indirect immunofluorescence on smears of washed and methanol-fixed mammalian spermatozoa gave poor results, due to high non-specific background staining, and a lack of correlation of immunofluorescence detected on the sperm surface with sperm surface immunoglobulins detected with sperm agglutination and sperm immobilisation tests (see Francavilla et al., 1987). Räsänen et al. (1992) have suggested that fixation may alter mammalian sperm surface antigens and influence immunofluorescent staining of spermatozoa. Furthermore, unfixed sperm smears produced negative results (see Francavilla et al., 1987). However, Francavilla et al. (1987) reported good correlation between sperm surface immunoglobulins detected by immunofluorescence and immunoglobulins detected using sperm agglutination tests, using an indirect immunofluorescence technique on unfixed human spermatozoa in suspension.

The lack of antichickens IgG binding to non-inseminated control spermatozoa indicates that chicken immunoglobulin observed on spermatozoa recovered from the vagina by lavage is of vaginal origin. Furthermore, the fact that $82.1 \pm 2.1\%$ of dead and only $7.7 \pm 3.4\%$ of living chicken spermatozoa recovered from the vagina by lavage 20 minutes following insemination had chicken immunoglobulin bound to their surface (see 3.5.4.2.), strongly suggests that the binding of chicken immunoglobulin to the sperm surface is closely related to sperm death.

The recovery of an average of $3.0 \pm 0.6 \times 10^6$ spermatozoa by vaginal lavage 20 minutes following insemination would represent around 10% of spermatozoa remaining in the vagina at that time. This, allied with the fact that spermatozoa inseminated intravaginally begin to reach the uterovaginal junction between 15 and 60 minutes following insemination (Allen & Grigg, 1957; Bobr et al., 1964b; Takeda, 1974), indicates that spermatozoa recovered from the vagina 20 minutes following insemination were likely to be representative of the vaginal sperm population as a whole.

Following the initial mass ejection of spermatozoa from the vagina, only approximately 6% of those remaining would ultimately be stored in the uterovaginal junction SSTs and potentially fertilise eggs. Thus, given the rapidity with which spermatozoa can traverse the vagina, some very efficient system must be responsible for the elimination of the other 94%. Takeda (1974) postulated that such sperm selection was due to sperm motility. However, in this study, spermatozoa treated with hypertonic buffer and neuraminidase exhibited reduced access in-vivo to the newly ovulated egg and uterovaginal junction SSTs respectively (see 3.4.1.3., 3.4.3.3.), although sperm integrity, and motility, were retained (see 3.4.1.1., 3.4.3.2.), indicating that the sperm selection process can be influenced without any observed change in motility at the point of insemination. Reduced motility would however, result from any oviducal sperm selection process,

as a result of sperm immobilisation, direct injury or cell death.

As previously mentioned (see 4.2.5.), the physical conditions within the chicken vagina are unlikely to stress spermatozoa unduly during transvaginal migration. However, given the anatomical relationship between the vagina, rectum and cloaca in domestic birds, an efficient mucosal immune system at the vagina to protect the oviduct against potentially pathogenic microorganisms would seem a necessity, and a logical extension of this might be to eliminate inseminated spermatozoa on the basis of their surface antigenicity.

It would seem logical that if immunoglobulin binding of the sperm surface was an active element of the vaginal sperm selection process, then such an event would be expected to precede sperm death. Thus the percentage of spermatozoa with chicken immunoglobulin bound to their surface would be higher than the percentage staining dead at any given time following insemination, as was observed (see 3.5.4.1.).

It has previously been postulated that mammalian sperm surface-associated proteins are non-antigenic in the female reproductive tract and have a protective function, masking spermatozoa, as foreign cells, from the local oviducal immune system (see Johnson, 1973; Metafora et al., 1989). Immunological recognition of the sperm

surface by the vagina might then arise from incomplete coating of the sperm surface with epididymal proteins, or lack of masking of antigenic proteins e.g. with sialic acid (Toshimori et al., 1988, 1991). Sperm surface antigenic heterogeneity within normal semen samples has been reported for several mammalian species, as a result of probing with antisera raised against sperm surface components (Glassy et al., 1984; see Moore, 1990), or with antisperm antibodies (Lenzi et al., 1988). Similarly, Trummel et al. (1992) have demonstrated sperm surface antigenic heterogeneity in rainbow trout spermatozoa, using sperm-specific monoclonal antibodies. In this study, chicken immunoglobulin bound fairly uniformly to the surface of spermatozoa recovered from the vagina by lavage, over variable but clearly demarcated areas, indicating sperm surface antigenic heterogeneity. The variable segmented immunoglobulin binding observed, in conjunction with sperm surface heterogeneity detected using WGA and the antiserum raised against chicken ejaculated seminal plasma proteins (see 3.1.2., 3.1.3.), is indicative of the existence of subpopulations of chicken spermatozoa, based on the sperm surface.

Turkey spermatozoa inseminated into chicken hens produced similar results to those obtained from chicken spermatozoa, in that the percentage of spermatozoa recovered from the vagina at any given time with chicken immunoglobulin bound was greater than the percentage staining dead. However, turkey spermatozoa exhibited

maximum immunoglobulin binding and cell death considerably earlier than homologous chicken spermatozoa (see 3.5.4.1.), possibly due to the presentation of turkey-specific antigens.

The segmental binding of chicken immunoglobulin to chicken and turkey spermatozoa may be indicative of the restriction of individual sperm surface associated proteins to specific regions of the sperm surface, as has been observed in mammals (Hjort et al., 1982), and differences in the location of chicken immunoglobulin binding on chicken and turkey spermatozoa confirms antigenic differences between the sperm surfaces of the 2 species.

Human spermatozoa present an antigenically distinct surface to that of chicken spermatozoa (see 3.5.2.) (gerbil spermatozoa were untested). Following the logic applied to the performance of turkey spermatozoa in the chicken vagina, mammalian spermatozoa might be expected to bind chicken immunoglobulin more rapidly, as appeared to be the case (see 3.5.4.1.).

The increase with time of the percentages of vaginal spermatozoa with immunoglobulin bound to their surface and staining dead would appear logical, given that spermatozoa selected for by the vagina would migrate into the

uterovaginal junction, leaving dead or immobilised spermatozoa to be conveyed posteriorly through the vagina to the cloaca (Takeda, 1974). However, it remains to be elucidated whether all spermatozoa which stain dead are killed within the initial 20 minutes following insemination and subsequently removed from the vagina slowly over an extended period of time, or whether spermatozoa have varying abilities to endure the conditions of the vagina and are killed slowly over a period of time. The difference between the 2 scenarios becomes important if sperm surface heterogeneity is related to some other factor e.g. genetic fitness (Cohen, 1967, 1969, 1973, 1975), in which case the first scenario would appear more attractive, as slower elimination of spermatozoa exhibiting fewer surface abnormalities could allow some such spermatozoa the opportunity to populate the uterovaginal junction SSTs and subsequently to fertilise eggs.

In chicken hens, the uterovaginal junction is situated within the multiple folds of tissue comprising the uterovaginal sphincter (Bohr et al., 1965). Because this structure is held tight for most of the ovulatory cycle, only relaxing at oviposition (Bohr et al., 1965), it would seem unlikely that the introduction of 1.0 ml of fluid into the mid-vaginal region would remove spermatozoa already within the uterovaginal junction. It would seem more likely that spermatozoa recovered by vaginal lavage would have been removed from the vagina.

It is possible that some spermatozoa, upon reaching the uterovaginal junction, may return to the vagina. However, such numbers are likely to be very small, and at least some living spermatozoa recovered from the vagina during the first few hours following insemination are likely to have been resident within the vagina since insemination. If this is the case, then it would thus appear that some chicken spermatozoa can survive within the clearly hostile environment of the vagina for several hours, whereas others perish within minutes. This would suggest an active selection of spermatozoa by the vagina, rather than a passive process in which the vagina is equally hostile to all spermatozoa, with those first to reach the uterovaginal junction surviving.

Spermatozoa recovered from the uterovaginal junction and infundibulum following multiple intravaginal insemination did not generally exhibit chicken immunoglobulin on their surface (see 3.5.4.3.). It is thus possible that such spermatozoa may constitute a selected, antigenically distinct subpopulation, given that immunoglobulin reactions at a membrane surface are not typically reversible in physiological conditions (Cohen & Werrett, 1975) or by repeated washing (Haas & D'Cruz, 1987). However, spermatozoa recovered from the uterovaginal junction and infundibulum following multiple intravaginal insemination exhibited reduced WGA staining (see 3.6.1.). Such reduced staining could have resulted from the loss of

sperm surface carbohydrate residues, to which immunoglobulin could have been bound.

Spermatozoa recovered from the uterovaginal junction and infundibulum approximately 4 hours following intrauterine insemination did not generally exhibit immunoglobulin on their surface (see 3.5.4.3.), although immunoglobulins of all classes are secreted into the oviducal lumen at these locations (Kimijima et al., 1990), suggesting that regions of the oviduct including and anterior to the uterovaginal junction are not involved in sperm selection. This would seem logical since, from an energy consideration, it would seem wasteful to store spermatozoa at the uterovaginal junction to subsequently eliminate them in the anterior oviduct.

Prior exposure of the chicken vagina to sperm surface antigens does not appear to influence binding of immunoglobulin to the sperm surface (see 3.5.4.1.), suggesting that sperm-binding immunoglobulins are not produced in a normal humoral response to sperm antigens.

There are several possible explanations for this apparent non-specific binding of immunoglobulin to the sperm surface. Parr and Parr (1985) have demonstrated the presence of several bacterial species in the mouse uterus, introduced by coitus. IgG and IgA specific to bacterial species are responsible for their agglutination in-vivo, but also cross-react with other bacterial species (Parr &

Parr, 1988). As previously mentioned (see 4.2.6.), the close anatomical relationship between the cloaca and the vagina of domestic birds is likely to result in the constant invasion of the vagina by various microorganisms of faecal origin. The vagina is therefore likely to possess an efficient mucosal immune system. Thus immunoglobulin secreted by plasma cells situated on the basal lamina of the vaginal epithelium (Kirk et al., 1989) may be in response to microbial antigens (see Cohen & McNaughton, 1974) and may interact non-specifically with molecules on the sperm surface which present antigenically similarly.

An alternative explanation for the apparent non-specificity of immunoglobulin interaction with the sperm surface might be that the immunoglobulin does not in fact interact directly with the sperm surface, but with molecules of microbial origin adsorbed onto the sperm surface. Copulation in domestic birds appears to be an awkward process, with semen deposition onto the mucosa of the everted vagina (Lake, 1981). The positions of the copulatory organs with respect to the cloaca in both sexes would allow potential microbial contamination of semen during ejaculation and transfer, and bacterial adherence to the mammalian sperm surface has previously been reported (see Aroux et al., 1991). However, examination of smears of spermatozoa recovered from the vagina by lavage following intravaginal insemination failed to reveal the presence of microorganisms adhering to the

sperm surface. Nevertheless, there is likely to be a constant turnover of very high numbers of microorganisms in the vagina, and it is possible that free microbial proteins are adsorbed onto the sperm surface, and that immunoglobulin subsequently interacts with these. The adsorption of such proteins might depend on the underlying sperm surface topography, thus giving rise to the segmental binding of immunoglobulin. It is however, questionable whether such proteins would be present in sufficient quantity to provide the extensive sperm surface coating observed, in the short period during which spermatozoa were exposed to them.

It is also possible that plasma cells in the vaginal mucosa produce immunoglobulin specific for antigens on the sperm surface, without sperm challenge, in a similar manner to those producing specific human ABO blood group agglutinins. The isoagglutinins anti-A and anti-B appear in human serum 3 to 6 months after birth and remain thereafter without any overt antigenic stimulation. The mechanism of their production is currently unknown, but it is believed that intestinal bacterial flora, which present similarly to the A and B antigens, may act as immunogens (Zaleski et al., 1983). Immunoglobulins interacting specifically with antigens on the sperm surface could thus be produced as a result of microorganisms in the vagina. Alternatively, it is known that immunoglobulin precursor lymphoblasts selectively migrate from the site of antigen challenge to localise in other mucosal tissues

(Bienenstock, 1981). Thus initial antigenic stimulation could occur at other mucosal sites.

If secreted immunoglobulins are not directed against spermatozoa, but are merely either cross-reacting with the sperm surface or with microbial proteins bound to the sperm surface, then an elimination of spermatozoa, based on sperm surface heterogeneity, would take place. However, a selection system based on a non-specific interaction is unlikely to select on any criterion of sperm quality. Conversely, a specific interaction between immunoglobulin and the sperm surface is far more likely to select spermatozoa on the basis of some aspect of sperm quality. Cohen (1967, 1969, 1973, 1975) has postulated that oviducal sperm selection may ultimately have a genetic basis, and that the extent of sperm wastage in the female reproductive tract is directly related to the number of chiasmata occurring during meiosis in the production of each spermatozoon. This postulation is supported by evidence for post-meiotic transcription from sperm DNA and the appearance and segregation of specific sperm antigens possibly coded from them (see Cohen & Gregson, 1978). However, there is a considerable body of evidence contradicting this hypothesis. Wallace (1974) produced evidence that chiasma frequency is not related to fertility, and Jacobs (1972) reported that around 2% of human conceptions arise from chromosomally abnormal spermatozoa. Furthermore, Mortimer (1978) argued that it is unlikely that the female reproductive tract would be

able to select against spermatozoa with minor defects such as chiasma faults, when spermatozoa with grossly abnormal genomes e.g. diploid (Mortimer, 1977), are allowed to reach the site of fertilisation.

There are several possible mechanisms working individually and/or jointly which may be responsible for sperm elimination from the vagina.

In addition to being found singly in washings, spermatozoa recovered from the vagina by lavage following intravaginal insemination were also observed in aggregates of variable size (see 3.5.4.1.). In some cases, spermatozoa were tightly agglutinated and in others appeared suspended in vaginal mucus. This, allied to the predominantly flagellar segmental binding of immunoglobulin observed, might suggest direct impairment of motility, due to restricted and/or abnormal flagellar activity and sperm-sperm agglutination (see Underdown & Schiff, 1986). However, although immunoglobulin binding to the sperm surface is unlikely to kill spermatozoa, most spermatozoa exhibiting immunoglobulin on their surface also stained dead, indicating the involvement of other, spermicidal processes.

Another possible mechanism of sperm elimination from the vagina is the activation of complement by sperm-bound IgG and/or IgA (see Underdown & Schiff, 1986; Bronson et al., 1987). A lytic complement cascade exists in the blood

serum of chickens (Koppenheffer, 1988), but has not yet been identified in the chicken oviduct. However, the lytic complement cascade has been identified in human cervical mucus (Price & Boettcher, 1979) and may be active within the mammalian oviduct (see D'Cruz et al., 1991). Various effects on mammalian sperm function in-vitro have been reported, including impairment of sperm motility (Bronson et al., 1987; D'Cruz et al., 1991), promotion of sperm immobilisation (Bronson et al., 1982) and phagocytosis (D'Cruz et al., 1991), and damage to acrosomal and plasma membranes (see D'Cruz et al., 1991). Furthermore, human seminal plasma is known to contain complement inhibitors (see D'Cruz et al., 1991).

Opsonisation of cells for phagocytosis is mainly mediated by IgG (Parr & Parr, 1988). There are various reports of the selective opsonisation of spermatozoa in the mammalian oviduct (e.g. Austin, 1960; Symons, 1967; Cohen & Werrett, 1975), accompanied by the induction of a massive leukocytosis within 30 minutes, and subsequent phagocytosis (Tyler, 1977; Phillips & Mahler, 1978; Pandya & Cohen, 1985; Cohen, 1988). However, although it remains a possibility, the inability in this study to detect phagocytes in vaginal mucosal smears obtained 2 hours following intravaginal insemination (see 3.5.4.1.) would suggest that phagocytosis is probably not an important sperm-eliminating mechanism in the chicken vagina.

Double mating experiments carried out in mammals, involving the recovery of spermatozoa from the anterior oviduct of one female and their subsequent insemination into a second female have indicated that such spermatozoa are extremely fertile compared with ejaculated spermatozoa (Cohen & McNaughton, 1974; Overstreet & Katz, 1977; Cohen & Tyler, 1980). Such an experiment would be difficult to expedite in the chicken, given that intravaginally inseminated spermatozoa are stored at the uterovaginal junction SSTs prior to their appearance in the anterior oviduct, and are believed to be released continuously, resulting in only very small numbers present in the oviducal lumen at any given time. Furthermore, the relatively large surface area of the chicken anterior oviducal lumen compared with that of laboratory mammals, would make efficient sperm recovery difficult. However, an in-vitro sperm selection system would potentially provide access to larger numbers of selected spermatozoa.

The vaginal mucosa clearly presents as a hostile environment towards spermatozoa in-vitro (see 3.5.4.4.), as it does in-vivo, unlike that of the infundibulum, which appears relatively benign. These results are generally in agreement with results obtained by Ashizawa and Nishiyama (1983), who reported poor maintenance of motility of chicken spermatozoa incubated in-vitro with vaginal tissue, compared with those incubated with infundibular or uterovaginal junction tissue. Similarly to the situation in-vivo (see 3.5.4.1.), the percentage of spermatozoa with

immunoglobulin bound to their surface exceeded the percentage staining dead, suggesting that the same mechanism is responsible for sperm death in-vitro and in-vivo.

Control spermatozoa and spermatozoa which had previously been incubated with vaginal mucosa in-vitro for one hour demonstrated similar abilities to gain access to the newly ovulated egg in-vivo (see 3.5.4.4.). Given the high percentage of spermatozoa staining dead after incubation with vaginal mucosa, the results obtained suggest that the surviving spermatozoa from the vaginal tissue incubation were more fertile than the control spermatozoa, as would be expected if they included a selected subpopulation.

4. 3. Spermatozoa in the Oviduct Prior to Fertilisation

4. 3. 1. Possible Effects of OPF on Spermatozoa In-Vivo

Mammalian spermatozoa require to capacitate in the female reproductive tract in order to gain fertilising ability (see Austin, 1951; Yanagimachi, 1981; Moore & Bedford, 1983). In most species capacitation takes place throughout the length of the oviduct and involves changes in sperm surface antigenicity (Koehler, 1978) and lectin staining (Koehler, 1978; Cross & Overstreet, 1987). Selective adsorption of proteins from uterine and oviducal fluids are also involved in sperm surface change (Voglmayr, 1987; Lippes & Wagh, 1989) and are believed to play important roles in events preceding fertilisation (Oliphant et al., 1984; Lippes & Wagh, 1989).

Spermatozoa from domestic birds are not believed to undergo capacitation prior to fertilisation (Howarth, 1971; Howarth & Palmer, 1972; Fujihara et al., 1973). Given that ovarian pocket fluid (OPF) may be involved in events preceding fertilisation in chickens (Ashizawa & Wishart, 1992), its effects on spermatozoa were investigated in-vitro.

Fluid collected from the ovarian pocket at around the time of ovulation clearly alters sperm surface antigenicity (see 3.6.2.), possibly as a result of adsorption of

antigens onto and/or loss from the sperm surface, and/or enzymic modification of existing antigens e.g. by loss of masking carbohydrate residues.

Staining of spermatozoa with FITC-conjugated WGA appeared unaffected by incubation with OPF (see 3.6.2.). However, spermatozoa were intensely stained and subtle changes were unlikely to be detected using epifluorescence microscopy.

The source of OPF in chickens is currently unknown but is unlikely to be the ovary, since fluid is not present in the follicles of laying hens and the follicular blood supply appears to be reduced at the time of ovulation (Phillips & Warren, 1937). Although OPF may be partly peritoneal in origin, another potential source is the oviduct. It has been reported that in many mammalian species, oviducal fluids flow into the peritoneal cavity, with maximal production occurring at the time of ovulation (Hamner, 1973). In chickens, the infundibulum becomes oedematous and active at the time of ovulation, particularly in the ovarian pocket adjacent to the mature follicle (Warren & Scott, 1934; Phillips & Warren, 1937). Fluid volume is maximal at this time, during which spermatozoa are most frequently found within the infundibulum and ovarian pocket (Bohr et al., 1964b; Morzenti et al., 1978).

Ashizawa and Wishart (1992) have postulated that OPF may represent the milieu in which fertilisation takes place,

thus suggesting that sperm surface changes observed in-vitro may occur naturally prior to fertilisation.

Howarth (1971), Howarth and Palmer (1972) and Fujihara et al. (1973), on the basis of results obtained from in-vitro fertilisation experiments, concluded that capacitation is not a requirement for spermatozoa from domestic birds to gain fertilising ability. However, Howarth (1983) demonstrated that testicular spermatozoa, which had not been exposed to epididymal secretions, could fertilise ova if inseminated intramagnally. Given that spermatozoa become coated with sperm surface-associated proteins and glycoproteins during transport through the excurrent duct system of the chicken (Esponda & Bedford, 1985), if sperm-egg interaction is a receptor-mediated process in chickens, as postulated by Howarth (1990), then it would seem logical that unmasking of sperm surface receptors would be necessary prior to fertilisation. There is evidence in mammals that sperm surface components directly involved in fertilisation are of testicular origin (Shalgi et al., 1990; Berger, 1990).

The acquisition of egg-binding sites as a consequence of the adsorption of OPF proteins onto the sperm surface would appear to be an attractive explanation for the results reported by Howarth (1983), allowing sperm-egg interaction without the prior unmasking of membrane components of testicular origin, and fertilisation of ova by both testicular and ejaculated spermatozoa. However,

the surface topography of testicular spermatozoa is likely to differ considerably from that of ejaculated spermatozoa, suggesting that adsorption of OPF proteins would require to be non-selective, and that such proteins would be weakly bound. It would seem likely that egg-binding molecules would require to be strongly bound to the plasma membrane and have direct contact with signal transduction machinery within the plasma membrane. Similarly, sperm surface-associated proteins are not strongly bound to the sperm surface (see 3.1.3.1.) and would seem unlikely to be egg-binding proteins, either masked or unmasked. Spermatozoa exhibited reduced uniformity of staining with antiserum raised against chicken seminal plasma proteins, after exposure to OPF, but the majority exhibited an enhanced staining intensity (see 3.6.2.). However, some spermatozoa showed a definite reduction in staining intensity, suggesting a loss of sperm surface associated proteins. A selective unmasking of testicular proteins could have occurred generally throughout the sperm population, but may not have been detected against the generally increased staining intensity, or possibly as a result of a lack of specificity of the antiserum.

Staining of spermatozoa, recovered from the uterovaginal junction and infundibulum, following multiple intravaginal insemination, with antiserum raised against seminal plasma proteins, did not reveal any oviduct-induced change in sperm surface antigenicity at around the time of ovulation

(see 3.6.1.), thus apparently confirming the finding by Morris et al. (1987) that chicken sperm surface antigenicity appears unchanged by sperm storage in the uterovaginal junction SSTs. These results would appear to contradict those obtained for spermatozoa previously incubated in-vitro with OPF (see 3.6.2.). However, it is questionable whether any meaningful comparison can be made between results obtained from the staining of spermatozoa recovered from the oviduct at around the time of ovulation, and 'unselected' spermatozoa incubated in-vitro with OPF. Generally, sperm numbers recovered from the anterior oviduct were very low compared with the numbers of spermatozoa used following in-vitro treatments, potentially resulting in differential staining between the 2 groups. This is likely to have been further influenced by the presence of oviducal tissue in oviducal sperm preparations. Furthermore, the infundibular SSTs are considerably smaller, shallower and less differentiated than their uterovaginal junction counterparts. Grigg (1957) suggested that release of spermatozoa from the infundibular SSTs might be due to mechanical pressure by the ovum while passing through the infundibulum. Thus spermatozoa recovered from the infundibular lumen could have been expelled from SSTs during tissue handling and washing and therefore may not have been exposed to OPF prior to staining.

Ashizawa and Wishart (1992) reported that OPF stimulated sperm motility in-vitro via 2 factors; calcium, and a low

molecular weight factor (0.2 kd). Although Ashizawa and Wishart (1992) measured sperm motility after only 15 seconds of incubation in OPF, much earlier than in this study, OPF was found in the present work, to have an almost immediate inhibitory effect on sperm motility, rendering spermatozoa almost immotile within 5 minutes. Spermatozoa appeared to remain otherwise viable (see 3.6.2.). Ashizawa and Wishart (1992) observed motility-stimulating effects at OPF working concentrations of up to 10%. However, if spermatozoa are bathed in this fluid prior to fertilisation, then the OPF concentration of 50% used in this study would seem more representative of the physiological concentration. Given that OPF contains approximately 2mM Ca^{2+} (Ashizawa and Wishart, 1992), a concentration stimulatory to sperm motility at 40 °C (Wishart & Ashizawa, 1987), the motility inhibition observed must have been due to some factor present in the fluid, rather than a lack of Ca^{2+} .

Although it is by no means clear that spermatozoa interact with OPF in-vivo prior to fertilisation, it is possible that the effects of OPF on sperm motility and sperm surface antigenicity observed in-vitro also occur in-vivo. If this is the case, then it would appear that OPF may unmask specific receptors on the sperm surface for the ovum and render fertilising spermatozoa immotile. The newly ovulated chicken ovum fills the entire lumen of the infundibulum, the site of fertilisation (Lake, 1984), and would therefore automatically encounter any spermatozoa

present there. There is no evidence to suggest that chicken spermatozoa require to be motile in order to fertilise ova in-vivo, and spermatozoa are believed to be passively transported through the anterior oviduct to the site of fertilisation (Fujii & Tamura, 1963; Van Krey et al., 1967).

If OPF is necessary for unmasking specific egg-binding proteins in-vivo prior to specific sperm-egg interaction, then it would seem necessary for a similar process to take place in-vitro. In the absence of OPF this might suggest a role for the inner perivitelline layer in-vitro, possibly via some diffusable factor. Diffusable egg factors have been implicated in fertilisation in some anuran amphibians (see Katagiri, 1987). However, the lack of any observable difference in the reaction of spermatozoa with WGA or antiserum raised against chicken ejaculated seminal plasma proteins, in the presence or absence of inner perivitelline layer (see 3.6.2.) would suggest the absence of any such effect. A possible explanation for this, as suggested previously (see 4.3.1.), is that specific unmasking of sperm surface proteins may not have been detected by the relatively insensitive probes used. A further possibility is that in an in-vitro system containing 20×10^6 spermatozoa in a fairly large volume of medium, only a very small proportion of spermatozoa entering into the close vicinity of the inner perivitelline layer would be affected by any diffusable factor. Subtle surface changes in a relatively

small number of spermatozoa are not likely to be detected against the general variability of sperm staining within any given sample.

4.3.2. Intraspecies Sperm-Inner Perivitelline Layer Interaction

It has been known for some time that the acrosome of chicken spermatozoa contains a trypsin-like enzyme (Langford & Howarth, 1974; Ho & Meizel, 1975; Brown & Hartree, 1976). The use of FITC-conjugated WGA to stain the inner perivitelline layer, a structure analogous to the mammalian zona pellucida (Howarth, 1990), allows quantitation of sperm acrosome reactions and provides a simple technique for the assay of sperm-inner perivitelline layer interaction (see 3.6.3.). Furthermore, this work confirms earlier reports by Ho and Meizel (1975), Bakst and Howarth (1977b) and Okamura and Nishiyama (1978) of hydrolysis of the inner perivitelline layer by acrosomal enzymes.

Bakst and Howarth (1977b) postulated that chicken spermatozoa do not undergo a mammalian or invertebrate type of acrosome reaction (Franklin, 1970; Yanagimachi, 1973), but reported a fenestrated appearance of the acrosomal membrane. However, in this study, spermatozoa were observed in association with hydrolysed areas of the inner perivitelline layer (see Figure 54) and at times

clearly lacking the acrosomal cap (not shown). These observations concur with those made by Koyanagi et al. (1988), who, using phase contrast and scanning electron microscopy, reported a lack of acrosomal caps on spermatozoa incubated in-vitro with inner perivitelline layer. It might be expected that spermatozoa from domestic birds would undergo a mammalian type acrosome reaction, given that mammalian type acrosome reactions are observed in lower vertebrates e.g. anuran amphibians (see Katagiri, 1987) and invertebrates e.g. echinoderms (see Longo, 1987).

Results obtained from the incubation of chicken spermatozoa with inner perivitelline layer from newly ovulated eggs and whole perivitelline layer from freshly laid eggs suggest that sperm-inner perivitelline layer interaction in domestic birds is a specific receptor mediated process (see Table 17), supporting the hypothesis advanced by Howarth (1990) of similarity with sperm-zona pellucida interaction in mammals (Storey et al., 1984; Bleil & Wassarman, 1983). A specific receptor-mediated process might be expected for domestic birds, given that a similar interaction has been shown to occur between spermatozoa and the vitelline coat of anuran amphibians (see Katagiri, 1987), and the vitelline envelope of echinoderms (see Longo, 1987). The outer perivitelline layer has been reported to be impenetrable by spermatozoa (Bakst & Howarth, 1977b). Thus acrosome reactions observed on whole perivitelline layer would necessarily

have occurred via non-receptor mediated sperm interaction with the inner aspect of the egg plasma membrane. Thus non specific sperm-egg interactions occur, but at a frequency of only approximately 7% of interactions occurring at the outer aspect of the inner perivitelline layer. Koyanagi et al. (1988) observed spontaneous chicken acrosome reactions in the absence of perivitelline layer.

The results obtained by Howarth (1971), Howarth and Palmer (1972) and Fujihara et al. (1973) do not necessarily preclude capacitation of chicken spermatozoa. Given that approximately 2×10^6 spermatozoa were layered directly over the germinal disc area of the ovum, then it would seem likely that fertilisation could take place as a result of non-specific sperm-egg interaction. Thus successful in-vitro fertilisations observed in these experiments could have been artefactual. This hypothesis receives further support from the fertility results obtained. Wishart (1987) reported the appearance of approximately 0.2% (99000 spermatozoa) of 60×10^6 intravaginally inseminated spermatozoa in the entire perivitelline layer of the first fertile egg, thus suggesting that the introduction of 2×10^6 spermatozoa directly over the germinal disc area may be excessive. Furthermore, out of 38 treated ova, Fujihara et al. (1973) reported only 11 successful fertilisations. Similarly, Howarth and Palmer (1972) obtained only 2 successfully fertilised ova from 5 incubated with spermatozoa. Howarth

(1971) observed similar fertilisation rates in-vitro and in artificially inseminated controls. However, the number of spermatozoa incubated with the ovum in-vitro was likely to have been considerably greater than the number of spermatozoa exposed to the first fertile egg in-vivo following intravaginal insemination. Thus fertilisation in-vivo was likely to have been considerably more efficient than in-vitro.

The dramatic reduction in sperm-inner perivitelline layer interaction during a 5 minute incubation in the presence of OPF (see Table 17), clearly suggests that sperm-inner perivitelline layer interaction in-vitro is motility-dependent, possibly in contrast to the situation in-vivo.

Chicken spermatozoa previously incubated with sonicated preparations of inner or whole perivitelline layer subsequently exhibited drastically reduced interaction with the inner perivitelline layer (see Table 19). The observation that spermatozoa remained motile throughout both incubations suggests prevention of interaction with the inner perivitelline layer as a result of the adsorption of egg-binding proteins from sonicated preparations, thus providing further support for the hypothesis that sperm-egg interaction in domestic birds is a specific process. Similar results were obtained by Howarth (1990), who, using spermatozoa at a concentration of $250 \times 10^6/\text{ml}$ observed that during a 60 minute incubation following sperm exposure to solubilised inner

perivitelline layer, inner perivitelline layer sheets were not fragmented. However, no estimation of sperm motility was made during either of the incubations. Furthermore, the results obtained in this study allowed quantitation of the effect of pre-incubation with sonicated inner perivitelline layer proteins, rather than the purely qualitative information provided by the earlier study.

Results obtained from the incubation of chicken spermatozoa with inner perivitelline layer from early or intermediate follicular ova (see Table 17) suggests that sperm-binding proteins are present and functional at a fairly early stage of oocyte development, and concur with results from previous studies in vertebrates and invertebrates, which have generally indicated the ability of spermatozoa to penetrate oocytes at early stages of maturation (see Longo, 1987).

The concentration of acrosome reactions on the inner perivitelline layer from the animal and vegetal poles did not differ significantly (see Table 17), indicating a lack of preferential sperm-inner perivitelline layer interaction at either pole. These results would appear to contradict those of Howarth and Digby (1973), Ho and Meizel (1975) and Bakst and Howarth (1977b), who reported the preferential hydrolysis of the region of inner perivitelline layer overlying the germinal disc. However, preferential hydrolysis reported by Ho and Meizel (1975) was based on the rate at which filter papers soaked in

chicken sperm acrosomal extract hydrolysed the inner perivitelline layer of an intact mature follicular egg in-vitro, and highlighted possible differences in inner perivitelline layer structure or in composition and structure of underlying material between the animal and vegetal poles, rather than differences in sperm binding affinity. Bakst and Howarth (1977b) reported the preferential hydrolysis of the inner perivitelline layer at the animal pole, following a 15 minute incubation of newly ovulated eggs with spermatozoa in-vitro. From these results, they postulated the existence of a concentrated array of sperm-binding proteins associated with the inner perivitelline layer at the animal pole. However, their assessment of inner perivitelline layer damage was qualitative rather than quantitative and may also have reflected an increased susceptibility of the perivitelline layer at the animal pole to hydrolysis, rather than an increased sperm-binding affinity. Furthermore, SDS-PAGE might be expected to reveal any significant increase in sperm-binding proteins at the animal pole. However, in this study, no difference was observed in the relative concentrations of perivitelline layer proteins from the animal or vegetal poles (see 3.6.3.1.).

Given that fertilisation takes place within the germinal disc of the animal pole, it would seem illogical that spermatozoa should have equal access to the inner perivitelline layer at the animal and vegetal poles. Howarth and Digby (1973) reported the rupture of the

inner perivitelline layer overlying the germinal disc area, following the incubation of intact newly ovulated eggs with spermatozoa, and postulated the chemotactic attraction of spermatozoa to the germinal disc region. Although their results could be interpreted similarly to those obtained by Ho and Meizel (1975), chemotaxis might explain the lack of preferential hydrolysis of inner perivitelline layer from the animal pole in this study. It is likely that any chemotactic stimulus released by the animal pole of the intact egg would be absent or severely diminished in the in-vitro system used in this study.

4. 4. Relative Importance of the Vagina and Inner Perivitelline Layer as Barriers to Interspecies Fertilisation

4. 4. 1. Interspecies Sperm-Inner Perivitelline Layer Interaction

Turkey, guinea fowl and duck spermatozoa hydrolysed chicken inner perivitelline layer in-vitro (see Table 18), indicating incomplete species specificity at this level. This observation is further supported by the dramatic reduction in turkey sperm-chicken inner perivitelline layer interaction subsequent to incubation with sonicated chicken inner or whole perivitelline layer (see Table 19). However, chicken and guinea fowl inner perivitelline layers were hydrolysed preferentially by homologous spermatozoa, indicating a degree of species specificity, similar to that observed in closely related mammalian species (Fukuda et al, 1979). Differences in chicken inner perivitelline layer hydrolysis as a result of incubation with chicken or turkey spermatozoa may also have been due to a degree of species specificity, but differences in sperm motility between the 2 species may also have been partially responsible. Poor homologous and heterologous inner perivitelline layer hydrolysis by guinea fowl spermatozoa is likely to have been due to rapid loss of motility in-vitro following collection (J.P. Brillard, personal communication). Duck spermatozoa were observed to be vigorously motile prior to

heterologous incubations with inner perivitelline layer, thus poor hydrolysis of chicken and guinea fowl inner perivitelline layer may have been due to low affinity for heterologous sperm binding proteins. This might be expected, given that the duck belongs to a different order of birds (Anseriformes) from the chicken and guinea fowl, which are closely related members of the order Galliformes (Pycraft, 1931).

The results obtained from the incubation of chicken spermatozoa with chicken inner perivitelline layer, following incubation with sonicated chicken, quail or turkey whole perivitelline layer (see Table 19) indicate considerably less interaction of chicken spermatozoa with quail whole perivitelline layer proteins, than with chicken or turkey, given that the protein concentrations of sonicated preparations were similar. This would appear to indicate selectivity of sperm-perivitelline layer protein interaction, and suggests that chicken spermatozoa may recognise sperm-binding proteins on turkey ova more readily than on quail ova, implying that chicken spermatozoa may more readily fertilise turkey than quail ova.

Staining intensities of protein bands on electrophoretic profiles of chicken inner perivitelline layer were affected variably by incubation with washed chicken spermatozoa (see 3.6.3.1.), possibly suggesting a degree of selectivity in uptake by the sperm surface. Molecular

weights assigned in other studies to inner perivitelline layer proteins separated by SDS-PAGE have varied. Kido and Doi (1988) reported 3 major glycoprotein bands at 32, 183 and >1000 kD, whereas Howarth (1992) reported major bands at 33, 53.5 and >200 kD. The protein bands affected by absorption with spermatozoa in this study appear to coincide to some extent with the major inner perivitelline components described by both Kido and Doi (1988) and Howarth (1992). The band at 180 kD in this study may be the same band as that described at 183 kD by Kido and Doi (1988), whereas the band at >200 kD may be the band described at >200 kD by Howarth (1992). The discrepancy between the studies of Kido and Doi (1988) and Howarth (1992), in the molecular weight assigned to the lower molecular weight band may indicate different proteins or different states of the same protein. The band at 44 kD in this study could be the band described by Kido and Doi (1988) at 32 kD, or that described by Howarth (1992) at 53.5 kD.

Thus, if absorption of major inner perivitelline layer glycoproteins is selective, then it is possible that all 3 major glycoprotein components of the chicken inner perivitelline layer are involved in fertilisation. In mammals, generally only ZP3 (Sacco et al., 1984, 1986) or ZP3 and ZP2 (see Wassarman, 1992) bind spermatozoa. If inner perivitelline layer proteins interact specifically with unmasked sperm surface proteins, then the removal of sperm surface associated proteins by washing prior to

incubation with sonicated preparations, and their continued loss during incubation were likely to enhance the selective uptake of sperm-binding proteins. The absence of the protein band at 180 kd and drastically reduced staining intensity of the band at >200 kd from absorbed chicken whole perivitelline layer, would appear to further support the idea of selective protein adsorption, given the relatively unaffected staining intensities of bands at 13 and 80 kd. However, the reduction in staining intensity of bands at 16 and 48 kd would suggest some non-selective uptake of proteins by the sperm surface. Furthermore, the absence of most high molecular weight protein bands from quail and turkey whole perivitelline layer profiles following absorption might suggest extensive non-selective uptake of protein. It would therefore seem likely that non-selective uptake of protein would have contributed to the reduction of sperm interaction with inner perivitelline layer following incubation with sonicated perivitelline layer preparations, particularly in cases where heterologous combinations were used.

4.4.2. Interspecies Differences in Sperm Surface and Seminal Plasma Components in Relation to Barriers to Interspecies Fertilisation

Lectin staining of chicken, quail, turkey, pigeon and duck spermatozoa revealed several interspecies differences in

sperm surface distribution of carbohydrate residues (see 3.7.2.), as has been observed for mammalian spermatozoa (Nicolson et al., 1977; Russell et al., 1983; Young et al., 1986). In all species studied, WGA was the major sperm-binding lectin, particularly so for chicken spermatozoa, indicating a predominance of N-acetylglucosamine-type residues (Young et al., 1986). Sialic acids are probably a major component (see 3.4.3.1. for chicken spermatozoa), given the variable patchy staining with the N^I, N^{II}, N^{III}-triacylchitotriose-specific lectin from Solanum tuberosum, of chicken, turkey, duck and pigeon spermatozoa. The carbohydrate character of the quail sperm surface appears to differ generally from the other species studied. Quail spermatozoa lack N^I, N^{II}, N^{III}-triacylchitotriose on their surface, but express α -D-mannosyl and/or α -D-glucosyl-type residues over their entire surface, whereas on the other species studied such residues appear restricted to the head and midpiece regions. Quail spermatozoa also exhibit α -L-fucosyl residues non-uniformly distributed over their entire surface. These residues are absent from the other species studied.

Interspecies sperm surface differences were further highlighted by variable reactivity with antiserum raised against chicken ejaculated seminal plasma proteins (see 3.7.1.). Spermatozoa from all species studied reacted with the antiserum, indicating the presence of epitopes similar to those on homologous spermatozoa. Homologous

spermatozoa, as would be expected, were generally more intensely stained than their heterologous counterparts. Esponda and Bedford (1985) reported a lack of staining of duck and pigeon spermatozoa recovered from the ductus deferens, with an antiserum raised against chicken epididymal and ductus deferens seminal plasma. In a later study, Morris et al. (1987) observed staining of turkey and quail spermatozoa to an extent similar to that observed on homologous spermatozoa, using the same antiserum. Differences in heterologous sperm staining between this study and those of Esponda and Bedford (1985) and Morris et al. (1987) may have been due to differences in the source of seminal proteins used for antiserum production.

The electrophoretic profiles of seminal plasmas from different avian species are clearly species specific, with few protein bands present at coincidental molecular weights in all species studied (see 3.7.3.1.). Some proteins in heterologous mammalian epididymal fluids exhibit similar amino acid sequences and have been shown to share molecular weights and antigenic determinants (Rankin et al., 1992a). Furthermore, some such proteins have been shown to associate with the sperm surface and are believed to have similar functions in different species. For example, an 18 kd mouse epididymal protein (MEP 10; Rankin et al., 1992a) is believed to be the homologue of proteins B and C, two retinoic acid-binding proteins present in rat cauda epididymal fluid (see Rankin

et al., 1992b). However, in this study, seminal plasma proteins with coincidental molecular weights in all species were stained at varying intensities and generally exhibited variable antigenicity between species, suggesting the unlikelihood of common function. Exceptions were the strongly stained protein bands at 66 and 75-80 kd in chicken, turkey, duck and quail profiles, which generally appeared similar and may have similar functions. Results from this study (see 3.1.4.1.) and that of Morris et al. (1987) indicate that neither of these proteins associates with the sperm surface, and are suggesting sperm-supportive roles, e.g. as carrier molecules, similar to some albumin-like proteins in human seminal plasma (Orlando et al., 1988). The intensity of immunological staining of pigeon seminal plasma proteins did not reflect the intense sperm surface staining observed. This could have been due to poor transfer of some polypeptides from the gel to the Immobilon membrane, failure of polypeptides to bind to Immobilon, or denaturation of polypeptides by SDS, altering their antigenicity (Cumming & Burgoyne, 1985). There is also the possibility that proteins adopt different conformations in seminal plasma and on the sperm surface. Such results emphasise the lack of reliability in correlating antigenic data obtained from Western blots following SDS-PAGE, with proteins in-situ on the sperm surface, or in their native state.

O'Rand (1988) has postulated that for mammals, species specificity at the level of sperm-egg interaction may be dependent on relatedness of species.

Of the Galliformes, turkeys (family phasianidae, sub-family meleagrinae) are more distantly related to chickens than are quail (both family phasianidae, sub-family phasiinae) (Pycraft, 1931). However, in-vitro, chicken spermatozoa appear to interact with turkey more readily than with quail inner perivitelline layer components (see Table 19), suggesting that species-specificity at the level of sperm-egg interaction may not be directly related to species divergence within the order Galliformes. Furthermore, the turkey sperm surface appears to share more antigenic groups with that of the chicken (as do those of duck and pigeon, which belong to different taxonomic orders), than does the quail, and turkey seminal plasma proteins are recognised immunologically by antiserum raised against chicken ejaculated seminal plasma proteins to a greater degree than are those from quail. Such findings might suggest that spermatozoa from the more distantly related duck (Anseriformes) and pigeon (Columbiformes) species are likely to outperform quail spermatozoa in the chicken vagina. However, it would appear that differences in surface characteristics between turkey and chicken spermatozoa are sufficient to allow oviducal selection for homologous spermatozoa (see 3.5.3.3.). Thus the hypothesis that duck or pigeon spermatozoa would

outperform quail spermatozoa in the chicken vagina would be difficult to test, and would be compounded by the rapid loss of quail sperm motility in-vitro following collection (Ogasawara & Huang, 1963).

Passage of spermatozoa through the female reproductive tract is believed to play an important role in the specificities of interspecies fertilisation in mammals (see O'Rand, 1988). In experiments with domestic birds, Ogasawara and Huang (1963) reported a fertilisation rate of 13% in eggs obtained from quail hens over a 12 day period following intravaginal insemination with jungle fowl semen. However, intrauterine insemination resulted in a fertilisation rate of 37% over the same period, thus supporting the finding in this study that the vagina is primarily responsible for interspecies sperm selection. McFarquhar and Lake (1964) suggested that relatively large volumes of chicken semen used in such cross-inseminations may result in overspill of intravaginally inseminated spermatozoa into the posterior uterovaginal junction. Such spermatozoa would avoid vaginal sperm selection and probably increase the fertilisation rate, thus providing an explanation for the apparently high fertilisation rate from intravaginal insemination. Furthermore in this study, small numbers of turkey spermatozoa were observed in chicken uterovaginal junction SSTs following intravaginal insemination (see 3.5.3.3.). Considerably greater numbers of spermatozoa ($100-250 \times 10^6$) were inseminated intravaginally in the experiments of Ogasawara

and Huang (1963), potentially leading to greater numbers of intravaginally inseminated spermatozoa surviving the vaginal sperm selection mechanism.

It seems likely that incomplete species specificity occurs at the level of sperm-egg interaction in domestic birds. Furthermore, the extent of ovum interaction with heterologous spermatozoa may not be dependent on the degree of relatedness for species within an order, such as the Galliformes. However, results obtained from the interaction of duck (Anseriformes) spermatozoa with inner perivitelline layer from Galliforme eggs (see Table 18) suggest an order-dependent specificity at the level of sperm-egg interaction.

SUMMARY

SUMMARY

During their transport through the excurrent duct system, chicken spermatozoa appear to undergo a post-testicular maturation process similar to that observed in mammals, involving the association of secreted epididymal proteins and glycoproteins with the sperm surface.

SDS-PAGE of anterior and posterior ductus deferens seminal plasma and sperm surface washings obtained from hypertonic buffer (1.0M NaCl) treatment of spermatozoa from the anterior and posterior ductus deferens, has indicated that protein association with the sperm surface is complete at the anterior ductus deferens, although secretion of some seminal plasma proteins continues throughout the ductus deferens. Furthermore, evidence has been obtained for the glycosylation, deglycosylation and antigenic change of sperm surface-associated and seminal plasma proteins in the ductus deferens and ampulla. Selective loss of proteins from the sperm surface is also evident in the ductus deferens.

A number of proteins were removed from the ejaculated sperm surface by exposure to hypertonic buffers (containing 0.20-0.40M NaCl). The molecular weights of extracted proteins after SDS-PAGE coincided with some of those of seminal plasma proteins, but their relative

staining intensities in electrophoretic profiles differed from those of their counterparts in seminal plasma, and some seminal plasma proteins were absent from extracted protein electrophoretic profiles, indicating selective protein uptake from seminal plasma by spermatozoa. However, individual sperm surface-associated proteins exhibited a range of affinities for the sperm surface, suggesting that protein association with the sperm surface may not be specific. Some sperm surface-associated proteins were removed in an NaCl concentration-dependent manner. Sperm access to the uterovaginal junction SSTs and newly ovulated egg in-vivo were also reduced in an NaCl concentration-dependent manner, but, at NaCl concentrations of between 0.15 and 0.25M, without any significant loss of sperm integrity in-vitro. Spermatozoa treated with 40 I.U./ml neuraminidase also exhibited reduced access to the uterovaginal junction SSTs in-vivo without significant loss of sperm integrity in-vitro. Furthermore, simply centrifuging or washing spermatozoa resulted in significant reductions in the ability of spermatozoa to gain access to the newly ovulated egg in-vivo, without adversely affecting sperm integrity in-vitro. These results clearly indicate a role for sperm surface-associated proteins and sperm surface sialic acid in vaginal sperm transport.

Exposure of ejaculated spermatozoa to glycerol at room temperature in concentrations lower than used as a

cryoprotectant resulted in a glycerol concentration-related reduction in sperm motility. This was accompanied by removal of sperm surface-associated proteins, in some cases also in a glycerol concentration-related manner, although glycerol appeared to prevent the removal of some sperm surface-associated proteins. Given the importance of sperm surface-associated proteins and sperm motility in vaginal sperm transport, it is possible that the glycerol-induced effects observed in this study may be involved in the contraceptive effect of glycerol in the chicken vagina in-vivo.

Incubation of spermatozoa from several mammalian and avian species with quail uterovaginal junction SSTs in-vitro has indicated that, contrary to previous reports, the uterovaginal junction SSTs are not selective with regard to the spermatozoa that enter them. Turkey spermatozoa, which present a surface antigenically different to chicken spermatozoa, but exhibit similar morphology and motility to chicken spermatozoa, populated chicken uterovaginal junction SSTs after uterovaginal junction insemination but not after intravaginal insemination, thus identifying the vagina as a major site of oviducal sperm selection.

A high percentage of chicken spermatozoa recovered from the chicken vagina by lavage 20 minutes after intravaginal insemination were found to have immunoglobulin (IgG and/or IgA) bound to their surface, with a slightly lower

percentage staining dead, and both percentages increasing with time following insemination. Similar results were obtained after insemination of turkey spermatozoa into chicken hens, but the percentages of recovered spermatozoa displaying immunoglobulin on their surface and staining dead increased to maxima over a shorter time period than that observed for homologous spermatozoa. The use of propidium iodide as a vital stain in conjunction with FITC-conjugated antichickens IgG showed a clear association between immunoglobulin binding to the chicken sperm surface and cell death. However, chicken spermatozoa recovered from the anterior regions of the chicken oviduct following multiple intravaginal inseminations did not display immunoglobulin on their surface.

Furthermore, incubation of spermatozoa with vaginal mucosa in-vitro resulted in immunoglobulin binding and loss of viable spermatozoa without any apparent adverse effect on the ability of spermatozoa to reach the site of fertilisation following intravaginal insemination, when compared with untreated control spermatozoa. This suggests that spermatozoa which remained viable following in-vitro incubation were the same as those selected in-vivo, and thus that a true sperm selection occurs in the vagina of the chicken hen, with an immunological basis.

Spermatozoa in vaginal washings obtained from previously virgin hens and hens which had been inseminated a number of times over preceding months showed similar staining after exposure to FITC-conjugated antichickens IgG, suggesting that oviducal sperm selection may not occur as a result of a humoral immune response to spermatozoa. Spermatozoa inseminated intrauterinely did not generally bind immunoglobulin in the uterovaginal junction or infundibulum, suggesting that the anterior oviduct may not be involved in sperm selection.

Exposure of chicken spermatozoa to ovarian pocket fluid in-vitro resulted in antigenic modification of the sperm surface and a dramatic reduction in sperm motility. It has recently been postulated that ovarian pocket fluid may be the milieu in which fertilisation takes place. If this is the case, then chicken spermatozoa may naturally undergo oviduct-induced changes prior to fertilisation.

Quantitation of acrosome reactions on the WGA-stained inner and whole perivitelline layer following incubation with chicken spermatozoa in-vitro has indicated that sperm-egg interaction in domestic birds is probably a specific receptor-mediated process similar to that observed in mammals. Furthermore, sperm-binding proteins were found to be present and functionally mature in early follicular eggs, but no evidence was obtained for

preferential attachment at the animal pole of the egg, as previously postulated.

Spermatozoa were observed to interact with heterologous inner perivitelline layer, but to a lesser extent than with homologous inner perivitelline layer, indicating limited species-specificity. Spermatozoa from the duck, which belongs to a different avian order (Anseriformes) to chicken, turkey and guinea fowl (Galliformes), exhibited only very limited interaction with chicken and guinea fowl inner perivitelline layer, suggesting a lack of recognition of sperm-binding proteins, related to species divergence. However, chicken spermatozoa interacted more readily with turkey than quail perivitelline layer proteins, suggesting, since the quail is a more closely related species to the chicken than is the turkey, that the relationship between extent of sperm-egg interaction and relatedness of species may not be as clear within the order Galliformes.

Furthermore, a range of lectins bound with similar affinity to chicken, turkey, pigeon and duck spermatozoa, from 3 different taxonomic orders, but exhibited a different affinity for spermatozoa from the quail, which is a species closely related to the chicken and turkey. These results indicate a lack of proportionality of sperm surface change with divergence of species. However, studies of heterologous sperm-inner perivitelline layer

interactions and transport of turkey spermatozoa through the chicken vagina indicate that the vagina is likely to present a more efficient barrier to interspecies fertilisation than does the inner perivitelline layer of the egg.

FUTURE WORK

FUTURE WORK

The work carried out in this study has contributed to the characterisation of post-testicular maturation, sperm-oviduct interaction and sperm-egg interaction in domestic birds. However, many of the fundamental mechanisms involved remain unsolved.

Further work will be required to determine the epididymal sources of individual sperm surface-associated proteins and to chart their distributions on the sperm surface. There is some evidence in mammals for capacitation-associated migration of sperm surface antigens believed to be involved in fertilisation (see Jones, 1990). It would be interesting to determine whether a similar process takes place on spermatozoa from domestic birds. Successful reassociation of extracted sperm surface-associated proteins with the sperm surface, accompanied by restoration of sperm access to the site of fertilisation, comparable to that of control spermatozoa, would demonstrate beyond any doubt, the importance of sperm surface-associated proteins in vaginal sperm transport.

Further work is necessary to determine the mechanisms involved in glycerol-induced extraction and protection of sperm surface-associated proteins, and the glycerol concentration-related reduction in sperm motility.

Repetition at avian body temperature of the glycerol experiment carried out in this study might provide a more accurate representation of in-vivo glycerol-induced effects on sperm function.

Further characterisation of the sperm selection mechanism in the vagina is necessary. This might include searching for complement components and implicating them in sperm death, and determination of the specificity of sperm-immunoglobulin interaction in the vagina. Furthermore, although it has been established in this study that the sperm surface is involved in vaginal sperm transport, it remains to be determined whether sperm surface-associated proteins and glycoproteins have a role in sperm storage in the uterovaginal junction SSTs.

At present, relatively little is known about sperm-egg interaction at molecular level in domestic birds. Although it has been shown that sperm-egg interaction is probably a specific, receptor mediated process, the identification and characterisation of sperm-binding proteins on the inner perivitelline layer and egg-binding proteins on the sperm surface, and the elucidation of the sequence of events prior to and during fertilisation are necessary pieces of work yet to be undertaken.

The advent of monoclonal antibodies has led to important advances in mammalian reproductive biology, allowing

determination of the distribution of individual antigens on the sperm surface and subsequent changes in their location correlating with post-testicular maturation (Villaroya & Scholler, 1986), and the localisation of the source of some proteins secreted by the epididymal epithelium (see Moore & Smith, 1988). Further examples of the utilisation of monoclonal antibodies include the identification of a sperm maturation-associated sialoglycoprotein with a cryptodeterminant, implicated in sperm protection from phagocytosis (Toshimori et al., 1988, 1991) and the location of sperm surface antigens involved in sperm-oolema fusion and the zona-induced acrosome reaction (see Jones, 1990).

The use of polyclonal antibodies in the study of molecular mechanisms is limited by their lack of specificity. The use of monoclonal antibodies may thus be necessary to determine the epididymal sources of individual sperm surface-associated proteins and their distributions on the sperm surface, and to identify any oviduct-induced changes to individual sperm surface-associated components prior to fertilisation or during sperm storage in the uterovaginal junction SSTs; and to identify which proteins in the inner perivitelline layer of the egg and on the sperm surface are involved in fertilisation.

REFERENCES

REFERENCES

Acott, T. S. and Hoskins, D. D. (1981) Bovine sperm forward motility protein: binding to epididymal spermatozoa. Biol. Reprod., 24: 234-240.

Alexander, N. J. and Anderson, D. J. (1987) Immunology of semen. Fertil. Steril., 47: 192-205.

Allen, G. J. and Bourne, F. J. (1978) Interaction of immunoglobulin fragments with the mammalian sperm acrosome. J. Exp. Zool., 203: 271-276.

Allen, T. E. and Bobr, L. W. (1955) The fertility of fowl spermatozoa in glycerol diluents after intrauterine insemination. Poult. Sci. 34: 167-169.

Allen, T. E. and Grigg, G. W. (1957) Sperm transport in the fowl. Aust. J. Agric. Res., 8: 788-799.

Aroux, M. R., Jacques, L. and Mathieu, D. (1991) Is the sperm bacterial ratio a determining factor in impairment of sperm motility: an in-vitro study in man with Escherichia coli. Int. J. Androl., 14: 264-270.

Asa, C.S. and Phillips, D.M. (1987) Ultrastructure of avian spermatozoa: a short review. In New Horizons in Sperm Cell Research, pp. 365-373 (Ed. H. Mohri). Japan Sci. Soc. Press, Tokyo.

Asch, R.H., Balmacada, J. and Paverstein, C.J. (1977) Failure of seminal plasma to enter the uterus and oviducts of the rabbit following artificial insemination. *Fertil. Steril.*, 28: 671-673.

Ashizawa, K. and Nishiyama, H. (1983) Prolonged survival of fowl spermatozoa in the oviducal tissues in organ culture. *Br. Poult. Sci.*, 24: 27-32.

Ashizawa, K., Ozawa, Y. and Okauchi, K. (1988) Changes of elemental concentrations around and on the surface of fowl sperm membrane during maturation in the male reproductive tract and after in-vitro storage. *Gamete Res.*, 21: 23-28.

Ashizawa, K. and Sano, R. (1990) Effects of temperature on the immobilisation and the initiation of motility of spermatozoa in the male reproductive tract of the domestic fowl, Gallus domesticus. *Compar. Biochem. Physiol.*, 96A: 297-301.

Ashizawa, K. and Wishart, G.J. (1992) Factors in fluid from the ovarian pocket that stimulate sperm motility in domestic hens. J. Reprod. Fert., In-press.

Austin, C.R. (1951) Observations on the penetration of the sperm into the mammalian egg. Aust. J. Sci. Res. Ser. B, 4: 581-596.

Austin, C.R. (1960) Fate of spermatozoa in the female genital tract. J. Reprod. Fert., 1: 151-156.

Austin, C.R. (1975) Sperm fertility, viability and persistence in the female tract. J. Reprod. Fert., Suppl. 22: 75-89.

Baker, R.R. and Bellis, M.A. (1988) 'Kamikaze' sperm in mammals? Anim. Behav., 36: 936-939.

Bakst, M.R. and Howarth, B. Jr. (1977a) The effect of glycerol and its removal on cock spermatozoa Concanavalin A and cationized ferritin binding sites. Poult. Sci., 56: 1318-1323.

Bakst, M.R. and Howarth, B. Jr. (1977b) Hydrolysis of the hen's perivitelline layer by cock sperm in-vitro. Biol. Reprod., 17: 370-379.

Bakst, M.R. and Howarth, B. Jr. (1977c) The fine structure of the hen's ovum at ovulation. *Biol. Reprod.*, 17: 361-369.

Bakst, M.R. (1978) Scanning electron microscopy of the vitelline membrane of the hen's ovum. *J. Reprod. Fert.*, 52: 361-364.

Bakst, M.R. (1980a) Luminal topography of the male chicken and turkey excurrent duct system. *Scan. Elect. Microsc.*, 3: 419-425.

Bakst, M.R. (1980b) Chicken and turkey oviductal pH at known times oviposition. *Poult. Sci.*, 59: 2793-2796.

Bakst, M.R. (1981) Sperm recovery from oviducts of turkeys at known intervals after insemination and oviposition. *J. Reprod. Fert.*, 62: 159-164.

Bakst, M.R. (1983) Fate of turkey spermatozoa after infundibular and intramaginal inseminations. *J. Reprod. Fert.*, 67: 315-317.

Bakst, M.R. and Richards, M.P. (1985) Concentrations of selected cations in turkey serum and oviductal mucosae. *Poult. Sci.*, 64: 555-563.

Bakst, M.R. (1987a) Anatomical basis of sperm storage in the avian oviduct. Scan. Microsc., 1: 1257-1266.

Bakst, M.R. (1987b) Quantification of intraepithelial lymphocytes in the turkey uterovaginal junction. Poult. Sci., 66: 2036-2038.

Bakst, M.R. and Bird, D.M. (1987) Localization of oviductal sperm-storage tubules in the american kestrel. The Auk, 104: 321-324.

Bakst, M.R. (1989) Oviducal storage of spermatozoa in the turkey: its relevance to artificial insemination technology. Br. Poult. Sci., 30: 441-447.

Ballas, S.K. (1981) Red cell membrane protein changes caused by freezing and the mechanisms of cryoprotection by glycerol. Transfusion, 21: 203-210.

Bardin, C.W., Shaha, C., Grima, J. and Cheng, C.Y. (1986) The use of Testibumen, a new marker for Sertoli cells, to study testicular physiology. Endocrinology, Suppl. 90: 90.

Barros, C. (1968) In-vitro capacitation of golden hamster spermatozoa with fallopian tube fluid of the mouse and rat. J. Reprod. Fert., 17: 203-206.

Bedford, J.M. (1965) Effect of environment on the phagocytosis of rabbit spermatozoa. J. Reprod. Fert., 9: 249-256.

Bedford, J.M. (1979) Evolution of the sperm maturation and sperm storage functions of the epididymis. In The Spermatozoon, pp. 1-21 (Eds. D.W. Fawcett and J.M. Bedford). Urban and Schwarzenberg, Baltimore.

Beer, A.E. and Neaves, W.B. (1978) Antigenic status of semen from the viewpoints of the female and male. Fertil. Steril., 29: 3-22.

Bekhtina, V.G. (1966) Morphological characters of polyspermic fertilization in domestic fowl. In Heritability and Variability in Domestic Fowls, pp. 92-109 (Ed. A. I. Fomin). Kolos, Moscow.

Bell, D.J. and Lake, P.E. (1962) A comparison of phosphomonoesterase activities in the seminal plasma of the domestic cock, turkey tom, boar, bull, buck rabbit and of man. J. Reprod. Fert., 3: 363-368.

Bellairs, R., Harkness, M. and Harkness, R.D. (1963) The vitelline membrane of the hen's ovum: a chemical and electron microscopical study. J. Ultrastruct. Res., 8: 339-359.

Berger, T. (1990) Changes in exposed membrane proteins during in-vitro capacitation of boar sperm. Mol. Reprod. Dev., 27: 249-253.

Bienenstock, J. (1981) The derivation, distribution and function of intestinal mucosal lymphocytes. Monogr. Allergy, 17: 233-249.

Blandau, R.J. (1969) Gamete transport - comparative aspects. In The Mammalian Oviduct, pp. 129-162 (Eds. E.S.E. Hafez and R.J. Blandau). Univ. Chicago Press.

Blaquier, J.A., Cameo, M.S., Stephany, D., Piazza, A., Tezon, J. and Sherins, R.J. (1987) Abnormal distribution of epididymal antigens on spermatozoa from infertile men. Fertil. Steril., 47: 302-309.

Bleil, J.D. and Wassarman, P.M. (1980) Mammalian sperm-egg interaction: identification of a glycoprotein in mouse zona pellucida possessing receptor activity for sperm. Cell, 20: 873-882.

Bleil, J.D. and Wassarman, P.M. (1983) Sperm-egg interactions in the mouse: sequence of events and induction of the acrosome reaction by a zona pellucida glycoprotein. Dev. Biol., 95: 317-324.

Blum, H., Beier, H. and Gross, H.J. (1987) Improved silver staining of plant proteins, RNA and DNA in polyacrylamide gels. Electrophoresis, 8: 93-99.

Bobr, L.W., Lorenz, F.W. and Ogasawara, F.X. (1964a) Distribution of spermatozoa in the oviduct and fertility in domestic birds. I. Residence sites of spermatozoa in fowl oviduct. J. Reprod. Fert., 8: 39-47.

Bobr, L.W., Ogasawara, F.X. and Lorenz, F.W. (1964b) Distribution of spermatozoa in the oviduct and fertility in domestic birds. II. Transport of spermatozoa in the fowl oviduct. J. Reprod. Fert., 8: 49-58.

Bobr, L.W., Lake, P.E., Lorenz, F.W., Ogasawara, F.X. and Krzanowska, H. (1965) Inhibition of ovulation in the domestic hen by intrauterine insemination. Poult. Sci., 44: 659-661.

Brackett, B.G. and Oliphant, G. (1975) Capacitation of rabbit spermatozoa in-vitro. Biol. Reprod., 12: 260-274.

Brillard, J.-P. and Bakst, M.R. (1990) Quantification of spermatozoa in the sperm storage tubules of turkey hens and the relation to sperm numbers in the perivitelline layer of eggs. Biol. Reprod., 43: 271-275.

Bronson, R.A., Cooper, G.W. and Rosenfeld, D.L. (1982) Correlation between regional specificity of antisperm antibodies to the spermatozoan surface and complement-mediated sperm immobilization. Am. J. Reprod. Immunol., 2: 222-224.

Bronson, R.A., Cooper, G.W. and Phillips, D.M. (1987) Effects of sperm reactive antibodies and complement on the ultrastructure of human spermatozoa. Ann. New York Acad. Sci., 513: 574-581.

Brooks, D.E. (1983) Selective binding of specific rat epididymal secretory proteins to spermatozoa and erythrocytes. Gamete Res., 7: 367-376.

Brooks, D.E. and Tiver, K. (1984) Analysis of surface proteins of rat spermatozoa during epididymal transit and identification of antigens common to spermatozoa, rete testis fluid and caudal epididymal plasma. J. Reprod. Fert., 71: 249-257.

Brown, C.R. and Hartree, E.F. (1976) Comparison of neutral proteinase activities in cock and ram spermatozoa and observations on a proacrosin in cock spermatozoa. J. Reprod. Fert., 46: 155-164.

Budras, K.D. and Sauer, T. (1975) Morphology of the epididymis of the cock (Gallus domesticus) and its effect upon the steroid sex hormone synthesis. I. Ontogenesis, morphology and distribution of the epididymis. Anat. Embryol., 148: 175-196.

Burke, W.H. and Ogasawara, F.X. (1969) Presence of spermatozoa in uterovaginal fluids of the hen at various stages in the ovulatory cycle. Poult. Sci., 48: 408-413.

Burke, W.H., Ogasawara, F.X. and Fuqua, C.L. (1969) Transport of spermatozoa to the site of fertilization in the absence of oviposition and ovulation in the chicken. Poult. Sci., 48: 602-608.

Burke, W.H., Ogasawara, F.X. and Fuqua, C.L. (1972) A study of the ultrastructure of the uterovaginal sperm-storage glands of the hen, Gallus domesticus, in relation to a mechanism for the release of spermatozoa. J. Reprod. Fert., 29: 29-36.

Burley, R.W. and Vadehra, D.V. (1989) The vitelline membrane. In The Avian Egg, pp. 147-170. Wiley Interscience.

Cartwright, E.J., Cowin, A. and Sharpe, P.T. (1992)
Surface heterogeneity of bovine sperm revealed by aqueous
two-phase partition. Biosci. Rep., 11: 265-273.

Cinader, B. and de Weck, A. (1976) Immunological response
of the female reproductive tract. WHO Workshop, 1975.
Scriptor, Copenhagen.

Clegg, E.D. (1983) Mechanism of mammalian sperm
capacitation. In Mechanism and Control of Animal
Fertilization, pp. 177-212 (Ed. J.F. Hartmann). Academic
Press, New York.

Cohen, J. (1967) Correlation between sperm 'redundancy'
and chiasma frequency. Nature, 215: 862-863.

Cohen, J. (1969) Why so many sperms? An essay on the
arithmetic of reproduction. Sci. Prog., 57: 23-41.

Cohen, J. (1973) Crossovers, sperm redundancy and their
close association. Heredity, 31: 408-413.

Cohen, J. and McNaughton, D.C. (1974) Spermatozoa: the
probable selection of a small population by the genital
tract of the female rabbit. J. Reprod. Fert., 39:
297-310.

Cohen, J. (1975) Gamete redundancy: wastage or selection? In Gametic Competition in Plants and Animals, pp. 99-112 (Ed. E. Mulcahy). Elsevier, New York.

Cohen, J. and Werrett, D.J. (1975) Antibodies and sperm survival in the female tract of the mouse and rabbit. J. Reprod. Fert., 42: 301-310.

Cohen, J. (1978) Non-specific (Fc) but selective attachment of antibody to sperms. Proc. 3rd Int. Symp. Immunol. Reprod., Sofia. pp. 234-238.

Cohen, J. and Gregson, S. (1978) Antibodies and sperm survival in the female genital tract. In Spermatozoa, Antibodies and Infertility, pp. 17-31 (Eds. J. Cohen and W.F. Hendry). Blackwell, London.

Cohen, J. and Tyler, K.R. (1980) Sperm populations in the female genital tract of the rabbit. J. Reprod. Fert., 60: 213-218.

Cohen, J. (1988) Double presentation of sperms to bitches and other mammals. In B.S.C.B. and B.S.D.B. Joint Spring Meeting Programme and Abstracts, p. 45.

Cooksey, E.J. and Rothwell, B. (1973) The ultrastructure of the Sertoli cell and its differentiation in the domestic fowl (Gallus domesticus). J. Anat., 114: 329-345.

Cooper, G.W., Overstreet, J.W. and Katz, D.F. (1979) Sperm transport in the reproductive tract of the female rabbit: motility patterns and the flagellar activity of spermatozoa. Gamete Res., 2: 35-42.

Cooper, T.G. (1986) The Epididymis, Sperm Maturation and Fertilization. Springer-Verlag, Berlin.

Cossu, G. and Boitani, C. (1984) Lactosaminoglycans synthesised by mouse male germ cells are fucosylated by an epididymal fucosyltransferase. Dev. Biol., 102: 402-408.

Cropp, C.S. and Schlaff, W.D. (1990) Antisperm antibodies. Arch. Immunol. Therap. Exp., 38: 31-46.

Cross, N.L. and Overstreet, J.W. (1987) Glycoconjugates of the human sperm surface: distributions and alterations that accompany capacitation in-vitro. Gamete Res., 16: 23-35.

Cumming, R. and Burgoyne, R.D. (1985) The immunoblotting technique for the characterisation of antibodies used in central nervous system immunocytochemistry. In Techniques in Immunocytochemistry, Vol. 3, pp. 55-78 (Eds. G.R. Bullock and P. Petrusz). Academic Press, London.

Cummins, J.M. and Yanagimachi, R. (1986) Development of ability to penetrate the cumulus oophorus by hamster spermatozoa capacitated in-vitro, in relation to the timing of the acrosome reaction. Gamete Res., 15: 187-212.

Dacheux, J.L. and Paquignon, M. (1980) Relation between the fertilizing ability, motility and metabolism of epididymal spermatozoa. Reprod. Nutr. Dev., 20: 1085-1099.

Davis, B.K., Byrne, R. and Bedigia, K. (1980) Studies on the mechanism of capacitation: albumin-mediated changes in plasma membrane lipids during in-vitro incubation of rat sperm cells. Proc. Nat. Acad. Sci. USA, 77: 1546-1550.

D'Cruz, O.J., Haas, G.G., Wang, B. and De Bault, L.E. (1991) Activation of human complement by IgG antisperm antibody and the demonstration of C3 and C5b-9-mediated immune injury to human sperm. J. Immunol., 146: 611-620.

Depeiges, A. and Dafaure, J.-P. (1983) Binding to spermatozoa of a major soluble protein secreted by the epididymis of the lizard Lacerta vivipara. Gamete Res., 4: 401-406.

Dickmann, Z. (1962) Experiments on interspecific sperm penetration through the zona pellucida. J. Reprod. Fert., 4: 121-124.

Dukelow, W.R., Chernoff, H.N. and Williams, W.L. (1967) Properties of decapacitation factor and presence in various species. J. Reprod. Fert., 14: 393-399.

Eddy, E.M., Vernon, R.B., Muller, C.H., Hahnel, A.C. and Fenderson, B.A. (1985) Immunodissection of sperm surface modifications during epididymal maturation. Am. J. Anat., 174: 225-237.

Edey, T.N., Thwaites, C.J., Piggot, F.A. and O'Shea, T. (1975) Fertility and sperm transport in Merino ewes at the first oestrous following embryonic death. J. Reprod. Fert., 43: 485-494.

Eng, L.A. and Oliphant, G. (1978) Rabbit sperm reversible decapacitation by membrane stabilisation with a highly purified glycoprotein from seminal plasma. Biol. Reprod., 19: 1083-1094.

Esponda, P. and Bedford, J.M. (1985) Surface of the rooster spermatozoon changes in passing through the wolffian duct. J. Exp. Zool., 234: 441-449.

Esponda, P. and Bedford, J.M. (1987) Post-testicular change in the reptile sperm surface with particular reference to the snake, Natrix fasciata. J. Exp. Zool., 241: 123-132.

Fayrer-Hosken, R.A. and Caudle, A.B. (1990) Bovine in-vitro fertilization: will the technique be practical? Embryo Transf., 5: 1-5.

Feuchter, F.A., Vernon, R.B. and Eddy, E.M. (1981) Analysis of the sperm surface with monoclonal antibodies: topographically restricted antigens appearing in the epididymis. Biol. Reprod., 24: 1099-1110.

Feuchter, F.A., Tabet, A.J. and Green, M.F. (1988) Maturation antigen of the mouse sperm flagellum. I. Analysis of its secretion, association with sperm, and function. Am. J. Anat., 181: 67-76.

Findlay, A.L.R. (1984) Reproduction and the Fetus. Edward Arnold, London.

Florman, H.M., Bechtol, K.B. and Wassarman, P.M. (1984) Enzymatic dissection of the function of the mouse egg's receptor for the sperm. *Dev. Biol.*, 106: 243-255.

Florman, H.M. and Wassarman, P.M. (1985) O-Linked oligosaccharides of mouse egg ZP3 account for its sperm receptor activity. *Cell*, 41: 313-324.

Fournier-Delpech, S., Hamamah, S., Colas, G. and Courot, M. (1983) Acquisition of zona binding structures by ram spermatozoa during epididymal passage. In The Sperm Cell, pp. 103-110 (Ed. J. Andre). Martinus Nijhoff, The Hague.

Fournier-Delpech, S. and Courot, M. (1987) Sperm-zona pellucida binding activity. In Oxford Reviews of Reproductive Biology, Vol. 9, pp. 294-331 (Ed. J.R. Clarke). Oxford University Press, Oxford.

Francavilla, F., Romano, R., Santucci, R., Catignani, P., Barone, A. and Fabbrini, A. (1987) Detection of sperm surface related antibodies by indirect immunofluorescence test on sperm suspensions. *Andrologia*, 19: 69-75.

Franklin, L.E. (1970) Fertilization and the role of the acrosomal region in non-mammals. *Biol. Reprod.*, Suppl. 2: 159-176.

Fraser, L.R. (1984) Mouse sperm capacitation in-vitro involves loss of a surface-associated inhibitory component. J. Reprod. Fert., 72: 373-384.

Freundl, G., Grimm, H.J. and Hoffman, N. (1988) Selective filtration of abnormal spermatozoa by the cervical mucus. Human. Reprod., 3: 277-280.

Friess, A.E., Sinowatz, F., Wrobel, K.H. and Scklekwinisch, R. (1978) The uterovaginal sperm host glands of the quail (Coturnix coturnix japonica). Cell Tissue Res., 191: 101-114.

Froman, D.P. and Thurston, R.J. (1984) Decreased fertility resulting from treatment of fowl spermatozoa with neuraminidase or phospholipase C. Poult. Sci., 63: 2479-2482.

Froman, D.P. and Engel, H.N. (1989) Alteration of the spermatozoal glycocalyx and its effect on duration of fertility in the fowl (Gallus domesticus). Biol. Reprod., 40: 615-621.

Fujihara, N., Tanaka, K. and Nishiyama, H. (1973) In-vitro fertilization of fowl ova. Jap. J. Zootech. Sci., 44: 564-568.

Fujihara, N., Koga, O. and Nishiyama, H. (1983) Secretory function of the ductus deferens of male chickens. Poult. Sci., 62: 557-560.

Fujii, S. (1963) Histological and histochemical studies on the oviduct of the domestic fowl with special reference to the region of uterovaginal juncture. Arch. Histol. Jap., 23: 447-459.

Fujii, S. and Tamura, T. (1963) Location of sperms in the oviduct of the domestic fowl with special reference to storage of sperms in the vaginal gland. J. Fac. Fish Anim. Husb., Hiroshima Univ., 5: 145-163.

Fujii, S. (1976) Scanning electron microscopical observation on the penetration mechanism of fowl spermatozoa into the ovum in the process of fertilization. J. Fac. Fish Anim. Husb., Hiroshima Univ., 15: 85-92.

Fukuda, Y., Maddock, M.B. and Chang, M.C. (1979) In-vitro fertilization of two species of deer mouse eggs by homologous or heterologous sperm and penetration of laboratory mouse eggs by deer mouse sperm. J. Exp. Zool., 207: 481-490.

Gallagher, J.T. (1984) Carbohydrate-binding properties of lectins: a possible approach to lectin nomenclature and classification. Biosci. Rep., 4: 621-632.

Gilbert, A.B. (1979) Female genital organs. In Form and Function in Birds, Vol. 1, pp. 237-360 (Eds. A.S. King and J. McLelland). Academic Press, New York.

Glassy, M.C., Surh, C.D. and Sarkar, S. (1984) Murine monoclonal antibodies that identify antigenically distinct subpopulations of human sperm. Hybridoma, 3: 363-371.

Goldstein, I.J. and Hayes, C.E. (1978) Lectins-carbohydrate binding proteins of plants and animals. Adv. Carbohydrate Chem. Biochem., 35: 128-340.

Gordon, M., Dandekar, P.V. and Bartoszewicz, W. (1975) The surface coat of epididymal ejaculated and capacitated sperm. J. Ultrastruct. Res., 50: 199-207.

Goto, K., Kajihara, Y., Kosaka, S., Koba, M., Nakanishi, Y. and Ogawa, K. (1988) Pregnancies after in-vitro fertilization of cow follicular oocytes, their incubation in-vitro and their transfer to the cow uterus. Theriogenology, 29: 251.

Graham, J.K., Kunze, E. and Hammerstedt, R.H. (1990) Analysis of sperm cell viability, acrosomal integrity and mitochondrial function using flow cytometry. Biol. Reprod., 43: 55-64.

Grigg, G.W. (1957) The structure of stored sperm in the hen and the nature of the release mechanism. Poult. Sci., 36: 450-451.

Haas, G.G. and D'Cruz, O. (1987) Inability of sperm washing to remove antisperm antibodies. J. Androl., 8: 21.

Hall, J.C., Hadley, J. and Doman, T. (1991) Correlation between changes in rat sperm membrane lipids, protein, and the physical state during epididymal maturation. J. Androl., 12: 76-87.

Hamilton, D.W. (1980) UDP-galactose: N-acetylglucosamine galactosyltransferase in fluids from rat testis and epididymis. Biol. Reprod., 23: 377-385.

Hammer, R.E., Pursel, V.G., Rexroad, C.E., Wall, R.J., Bolt, D.J., Ebert, K.M., Palmiter, R.D. and Brinster, R.L. (1985) Production of transgenic rabbits, sheep and pigs by microinjection. Nature, 315: 680-683.

Hammerstedt, R. H. and Graham, J. K. (1991)
Cryopreservation of poultry sperm: the enigma of glycerol.
Cryobiology, 29: 26-38.

Hamner, C. E. (1973) Oviducal fluid composition and physiology. In Handbook of Physiology, Section 7, Vol. 2, Female Reproductive Systems, Part 2, pp. 141-151 (Eds. R. O. Greap and E. B. Astwood). Am. Physiol. Soc., Washington.

Hanada, A. and Chang, M. C. (1976) Penetration of hamster and rabbit zona-free eggs by rat and mouse spermatozoa with special reference to sperm capacitation. J. Reprod. Fert., 46: 239-241.

Hanada, A. and Chang, M. C. (1978) Penetration of the zona-free or intact eggs by foreign spermatozoa and the fertilization of deer mouse eggs in-vitro. J. Exp. Zool., 203: 277-286.

Hancock, R. J. T. (1984) Immune responses to sperm: recent developments. In Immunological Aspects of Reproduction in Mammals, pp. 55-76 (Ed. D. B. Crighton). Butterworths, London.

Hanson, F.W. and Overstreet, J.W. (1981) The interaction of human spermatozoa with cervical mucus in-vivo. Am. J. Obstet. Gynecol., 140: 173-178.

Harlow, E. and Lane, D. (1988) Antibodies - A Laboratory Manual. Cold Spring Harbour Laboratory.

Harrison, R.A.P. and Vickers, S. (1990) Use of fluorescent probes to assess membrane integrity in mammalian spermatozoa. J. Reprod. Fert., 88: 343-352.

Hess, R.A., Thurston, R.J. and Biellier, H.V. (1976) Morphology of the epididymal region and ductus deferens of the turkey (Meleagris gallopavo). J. Anat., 122: 241-252.

Hess, R.A., Thurston, R.J. and Biellier, H.V. (1982) Morphology of the epididymal region of turkeys producing abnormal yellow semen. Poult. Sci., 61: 531-539.

Hjort, T., Ahuja, S.P. and Poulsen, F. (1982) Studies on sperm membrane antigens. In Immunological Factors in Human Reproduction, pp. 77-90 (Eds. S. Shulman, F. Dondero and M. Nicotra). Academic Press, London.

Ho, J. and Meizel, S. (1975) Hydrolysis of the hen egg vitelline membrane by cock sperm acrosin and other enzymes. J. Exp. Zool., 194: 429-438.

Holt, W.V. (1984) Membrane heterogeneity in the mammalian spermatozoa. Int. Rev. Cytol., 87: 159-189.

Howarth, B. Jr. (1971) An examination for sperm capacitation in the fowl. Biol. Reprod., 3: 338-341.

Howarth, B. Jr. (1971) Transport of spermatozoa in the reproductive tract of turkey hens. Poult. Sci., 50: 84-89.

Howarth, B. Jr. and Palmer, M.B. (1972) An examination of the need for sperm capacitation in the turkey, Meleagris gallopavo. J. Reprod. Fert., 28: 443-445.

Howarth, B. Jr. and Digby, S.T. (1973) Evidence for the penetration of the vitelline membrane of the hen's ovum by a trypsin-like acrosomal enzyme. J. Reprod. Fert., 33: 123-125.

Howarth, B. Jr. (1974) Sperm storage as a function of the female reproductive tract. In The Oviduct and its Functions, pp. 237-270 (Eds. A.D. Johnson and C.E. Foley). Academic Press, New York.

Howarth, B. Jr. (1983) Fertilizing ability of cock spermatozoa from the testis, epididymis and vas deferens following intramaginal insemination. Biol. Reprod., 28: 586-590.

Howarth, B. Jr. (1989) Influence of neuraminidase on fertility and on sperm storage in the hen's oviduct. Poult. Sci., 69: 119-123.

Howarth, B. Jr. (1990) Avian sperm-egg interaction: perivitelline layer possesses receptor activity for spermatozoa. Poult. Sci., 69: 1012-1015.

Howarth, B. Jr. (1992) Carbohydrate involvement in sperm-egg interaction in the chicken. J. Receptor Res., 12: 255-265.

Hsu, S.M. and Soban, E. (1982) Color modification of diaminobenzidine (DAB) precipitation by metallic ions and its application for double immunohistochemistry. J. Histochem. Cytochem., 30: 1079-1082.

Huang, T.T.F., Fleming, A.D. and Yanagimachi, R. (1981) Only acrosome-reacted spermatozoa can bind to and penetrate zona pellucida: a study using the guinea pig. J. Exp. Zool., 217: 287-290.

Hunter, R.H.F. (1974) Transport, migration and survival of spermatozoa in the female genital tract: species with intrauterine deposition of semen. In Sperm Transport, Survival and Fertilizing Ability in Vertebrates, Vol. 26, pp. 309-341 (Eds. E.S.E. Hafez and C.G. Thibault). INSERM, Paris.

Jacobs, P.A. (1972) Chromosome abnormalities and fertility in man. In The Genetics of the Spermatozoon, pp. 346-358 (Eds. R.A. Beatty and S. Gluecksohn-Waelsch). Depts. Genetics Univ. Edinburgh and Albert Einstein Coll. Med., Edinburgh and New York.

Jensen, C. (1969) Ultrastructural changes in the avian vitelline membrane during embryonic development. J. Embryol. Exp. Morph., 21: 467-484.

Johnson, M.H. (1973) Physiological mechanisms for the immunological isolation of spermatozoa. Adv. Reprod., 6: 279-323.

Jones, R., Pholpramool, C., Setchell, B.P. and Brown, C.R. (1981) Labelling of membrane glycoproteins on rat spermatozoa collected from different regions of the epididymis. Biochem. J., 200: 457-460.

Jones, R. (1989) Membrane remodelling during sperm maturation in the epididymis. In Oxford Reviews of Reproductive Biology, Vol. 11, pp. 285-337 (Ed. S. Milligan). Oxford University Press, Oxford.

Jones, R. (1990) Identifications and functions of mammalian sperm-egg recognition molecules during fertilisation. J. Reprod. Fert., Suppl. 42: 89-105.

Katagiri, C. (1987) Role of oviducal secretions in mediating gamete fusion in anuran amphibians. Zool. Sci., 4: 1-14.

Katz, D.F., Drobnis, E.Z. and Overstreet, J.W. (1989) Factors regulating mammalian sperm migration through the female reproductive tract and oocyte vestments. Gamete Res., 22: 443-469.

Kelm, S., Shukla, A.K., Paulson, J.C., Higa, H. and Schauer, R. (1986) Reconstitution of the masking effect of sialic acid groups of sialidase-treated erythrocytes by the action of sialyltransferases. Carbohydrate Res., 149: 59-64.

Khyse-Anderson, J. (1984) Electrophotting of multiple gels: a simple apparatus without buffer tank for rapid transfer of proteins from polyacrylamide to nitrocellulose. J. Biochem. Biophys. Meth., 10: 203-209.

Kibrick, S., Belding, D.L. and Merrill, B. (1952) Methods for the detection of antibodies against mammalian spermatozoa. I. A modified macroscopic agglutination test. Fertil. Steril., 3: 419-429.

Kido, S., Janado, M. and Nunoura, H. (1975) Macromolecular components of the vitelline membrane of hen's egg. I. Membrane structure and its deterioration with age. J. Biochem. (Tokyo), 78: 261-268.

Kido, S. and Doi, Y. (1988) Separation and properties of the inner and outer layers of the vitelline membrane of hen's eggs. Poult. Sci., 67: 476-486.

Kijimoto-Ochiai, S., Katagiri, Y.U. and Ochiai, H. (1985) Analysis of N-linked oligosaccharide chains of glycoproteins on nitrocellulose sheets using lectin-peroxidase reagents. Analyt. Biochem., 147: 222-229.

Kimijima, T., Hashimoto, Y., Kitagawa, H., Kon, Y. and Sugimura, M. (1990) Localization of immunoglobulins in the chicken oviduct. Jap. J. Vet. Sci., 52: 299-305.

Kirk, T.A., Van Krey, H.P., Hulet, R.M., Dunnington, E.A. and Denbow, D.M. (1989) The relationship of infertility to antibody production in the uterovaginal sperm storage tubules of turkey breeder hens. Theriogenology, 31: 955-961.

Koehler, J.K. (1978) The mammalian sperm surface: studies with specific labelling techniques. Int. Rev. Cytol., 54: 73-108.

Koppenheffer, T.L. (1988) Homologous and heterologous antibodies exhibit different requirements for activation of chicken complement. Am. Zool., 28: 59.

Koyanagi, F., Masuda, S. and Nishiyama, H. (1988) Acrosome reaction of cock spermatozoa incubated with the perivitelline layer of the hen's ovum. Poult. Sci., 67: 1770-1774.

Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T₄. Nature, 227: 680-685.

Lake, P.E. (1957) The male reproductive tract of the fowl. J. Anat., 91: 116-129.

Lake, P.E. (1966) Physiology and biochemistry of poultry semen. In Advances in Reproductive Physiology, Vol. I, pp. 93-123 (Ed. A. McLaren). Logos Press, London.

Lake, P.E. (1967) The maintenance of spermatozoa in the oviduct of the domestic fowl. In Reproduction in the Female Mammal, pp. 254-266 (Eds. G.E. Lamming and E.C. Amoroso). Butterworths, London.

Lake, P.E. and Hatton, M. (1968) Free amino acids in the vas deferens, semen, transparent fluid and blood plasma of the domestic rooster, Gallus domesticus. J. Reprod. Fert., 15: 139-143.

Lake, P.E., Smith, W. and Young, D. (1968) The ultrastructure of the ejaculated fowl spermatozoon. Q. J. Exp. Physiol., 53: 356-366.

Lake, P.E. (1975) Gametic production and the fertile period with particular reference to domesticated birds. Symp. Zool. Soc. London, 35: 225-244.

Lake, P.E. and Stewart, J.M. (1978) Artificial insemination in poultry. Bull. Min. Agric., Fish. Food, No. 213. HMSO, London.

Lake, P.E. (1981) Male Genital Organ in Form and Function in Birds, Vol. 2. (Eds. A.S. King and J. McLelland). Academic Press, London.

Lake, P.E., Ravie, O. and McAdam, J. (1981) Preservation of fowl semen in liquid nitrogen: application to breeding programmes. Br. Poult. Sci., 22: 71-77.

Lake, P.E. (1984) The male in reproduction. In The Physiology and Biochemistry of Domestic Fowl, Vol. 5, pp. 381-405. Academic Press, London.

Lake, P.E. and Wishart, G.J. (1984) Comparative physiology of turkey and fowl semen. In Reproductive Biology of Poultry, pp. 151-160 (Eds. F.J. Cunningham, P.E. Lake and D. Hewitt). Longman Group, Harlow.

Lake, P.E. (1986) The history and future of the cryopreservation of avian germ plasm. Poult. Sci., 65: 1-15.

Lake, P.E. and Ravie, O. (1987) Effect on fertility of low numbers of fowl spermatozoa inseminated in aqueous diluent or semen components of the fowl and turkey. Br. Poult. Sci., 28: 75-80.

Lake, P.E. and Ravie, O. (1988) Effect of trypsin and neuraminidase on fowl spermatozoa and their retention in the oviduct. Proc. 18th Wld. Poult. Congr., Nagoya, pp. 594-596.

Langford, B.B. and Howarth, B. Jr. (1974) A trypsin-like enzyme in acrosomal extracts of chicken, turkey and quail spermatozoa. Poult. Sci., 53: 834-837.

Lee, S. (1991) Biologists' involvement in the reproduction revolution. Biologist, 38: 85-88.

Lenzi, A., Morrone, S., Gandini, L., Lombardo, F. and Dondero, F. (1988) Cytofluometric study of antisperm antibodies directly bound to the sperm surface: evidences of the existence of various antigen sperm subpopulations. Am. J. Reprod. Immunol. Microbiol., 16: 65.

Leyton, L. and Saling, P.M. (1989a) 95 kd sperm proteins bind ZP3 and serve as tyrosine kinase substrates in response to zona binding. Cell, 57: 1123-1130.

Leyton, L. and Saling, P.M. (1989b) Evidence that aggregation of mouse sperm receptors by ZP3 triggers the acrosome reaction. J. Cell Biol., 108: 2163-2168.

Lippes, J. and Wagh, P.V. (1989) Human oviductal fluid (hOF) proteins. IV. Evidence for hOF proteins binding to human sperm. Fertil. Steril., 51:89-94.

Longo, F.J. (1987) Fertilization. Chapman and Hall, London.

Lorenz, F.W. and Ogasawara, F.X. (1968) Distribution of spermatozoa in the oviduct and fertility in domestic birds. VI. The relations of fertility and embryo mortality with site of experimental insemination. J. Reprod. Fert., 16: 445-455.

Maddock, M.B. and Dawson, W.D. (1974) Artificial insemination of deer mice (Peromyscus maniculatis) with sperm from other rodent species. J. Embryol. Exp. Morph., 31: 621-634.

Mann, T. (1964) The Biochemistry of Semen and the Male Reproductive Tract. Methuen, London.

Mann, T. and Lutwak-Mann, C. (1981) Male Reproductive Function and Semen. Springer-Verlag, Berlin.

Marquez, B.J. and Ogasawara, F.X. (1977) Ultrastructural changes in turkey spermatozoa after immersion in glycerolized media and during various steps used for cryopreservation. Poult. Sci., 56: 1806-1813.

Marx, J.L. (1988) Cloning sheep and cattle embryos. Science, 239: 463-464.

Mathis, G.F., Burke, W.H. and McDougald, L.R. (1983) Luteinizing hormone release and androgen production of avian hybrids in response to luteinizing hormone releasing hormone injection. Poult. Sci., 62: 715-717.

Mathis, G.F. and McDougald, L.R. (1987) Evaluation of interspecific hybrids of the chicken, guinea fowl and japanese quail for innate resistance to coccidia. Avian. Dis., 31: 740-745.

Mattner, P.E. (1966) Formation and retention of the spermatozoan reservoir in the cervix of the ruminant. Nature, 212: 1479-1480.

Mattner, P.E. (1968) The distribution of spermatozoa and leucocytes in the female genital tract in goats and cattle. J. Reprod. Fert., 17: 253-261.

Mattner, P.E. (1969) Differential leucocyte response to spermatozoa in the cervix and uterus of ewes. J. Reprod. Fert., 18: 297-303.

McFarquhar, A.M. and Lake, P.E. (1964) Artificial insemination in quail and the production of chicken-quail hybrids. J. Reprod. Fert., 8: 261-263.

McIndoe, W.M. and Lake, P.E. (1974) The distribution of some hydrolytic enzymes in the semen of the domestic fowl, Gallus domesticus. J. Reprod. Fert., 40: 359-365.

Menge, A.C. and Behrman, S.J. (1980) Immunological aspects of reproduction. In Human Reproduction: Contraception and Conception, pp. 488-505 (Ed. E. S. E. Hafez). Harper and Row, New York.

Menkveld, R., Franken, D.R., Kruger, T.F., Oehninger, S. and Hodgen, G.D. (1991) Sperm selection capacity of the human zona pellucida. Mol. Reprod. Dev., 30: 346-352.

Metafora, S., Porta, R., Ravagnan, G., Spera, G., Marchese, M., Furgi, A., D'Amello, I. and Peluso, G. (1989) Sperm immunoprotection in the process of mammalian reproduction. Symp. Unexplained Infert.: Basic Clin. Aspects, Rome., pp. 285-296.

Ministry of Agriculture, Fisheries and Food (1990)
Agriculture in the United Kingdom. H.M.S.O., London.

Moore, H.D.M. (1979) The net surface charge of mammalian spermatozoa as determined by isoelectric focussing. Changes following sperm maturation, ejaculation, incubation in the female tract and after enzyme treatment. *Int. J. Androl.*, 2: 449-462.

Moore, H.D.M. (1980) Localization of specific glycoproteins secreted by the rabbit and hamster epididymis. *Biol. Reprod.*, 22: 705-718.

Moore, H.D.M. and Bedford, J.M. (1983) The interaction of mammalian gametes in the female. In Mechanism and Control of Animal Fertilization, pp. 453-497 (Ed. J.F. Hartmann). Academic Press, London.

Moore, H.D.M. (1990) Development of sperm-egg recognition processes in mammals. *J. Reprod. Fert., Suppl.* 42: 71-78.

Morris, S.A. and Howarth, B. Jr. (1985) Effects of rooster sperm surface proteins on fertility in the hen. *Poult. Sci.*, 64 (Suppl. 1): 148.

Morris, S.A., Howarth, B. Jr., Crim, J.W., Rodriguez de Cordoba, S., Esponda, P. and Bedford, J.M. (1987) Specificity of sperm-binding Wolffian duct proteins in the rooster and their persistence on spermatozoa in the female host glands. J. Exp. Zool., 242: 189-198.

Mortimer, D. (1977) The survival and transport to the site of fertilization of diploid rabbit spermatozoa. J. Reprod. Fert., 51: 99-104.

Mortimer, D. (1978) Selectivity of sperm transport in the female genital tract. In Spermatozoa, Antibodies and Infertility, pp. 37-53. Blackwell Scientific Publications, Oxford.

Morton, D.B. and Glover, T.D. (1974) Sperm transport in the female rabbit: the effects of inseminate volume and sperm density. J. Reprod. Fert., 38: 139-146.

Morzenti, A., Ogasawara, F.X. and Fuqua, C.L. (1978) The relationship of infundibular fluid to the avian sperm transport mechanism. Poult. Sci., 57: 1173.

Munro, S.S. (1938a) The effect of testis hormone and the preservation of sperm life in the vas deferens of the fowl. J. Exp. Biol., 15: 186-196.

Munro, S.S. (1938b) Functional changes in fowl sperm during their passage through the excurrent ducts of the male. J. Exp. Zool., 79: 71-92.

Murphy, G.J.P. and Carroll, J. (1987) Detection and localization of antigens on the surface of mouse spermatozoa. Biochem. J., 241: 379-387.

Myles, D.G., Koppel, D.E., Cowan, A.E., Phelps, B.M. and Primakoff, P. (1987) Rearrangement of sperm surface antigens prior to fertilization. Ann. New York Acad. Sci., 513: 262-273.

Nakai, M., Hashimoto, Y., Kitigawa, H., Kon, Y. and Kudo, N. (1989) Histological study on seminal plasma absorption and spermiphagy in the epididymal region of domestic fowl. Poult. Sci., 68: 582-589.

Nash, S.R., Wiggins, M.E. and Birrenkot, G.P. (1986) In-vitro filling of the uterovaginal sperm host glands - timed entrance with avian and bull sperm. Poult. Sci., 65 (Suppl. 1): 97.

Neville, W.J., MacPherson, J.W. and Reinhart, B. (1971) The contraceptive action of glycerol in chickens. Poult. Sci., 50: 1411-1415.

Nicolson, G.L., Usui, N., Yanagimachi, R., Yanagimachi, H. and Smith, J.R. (1977) Lectin binding sites on the plasmamembranes of rabbit spermatozoa. Changes in surface receptors during epididymal maturation and after ejaculation. J. Cell Biol., 74: 950-962.

Nishiyama, H. and Ogawa, K. (1961) On the function of the vascular body, an accessory reproductive organ of the cock. Jap. J. Zootech. Sci., 32: 89-98.

Nishiyama, H., Ogawa, K. and Nakanishi, Y. (1968) The capacitation of the fowl spermatozoa. Mem. Fac. Agric., Kagoshima Univ., 6: 141-147.

Ogasawara, F.X. and Huang, R. (1963) A modified method of artificial insemination in the production of chicken-quail hybrids. Poult. Sci., 42: 1386-1392.

Ogasawara, F.X. and Lorenz, F.W. (1966) Early mortality in chicken embryos following intramaginal insemination. Poult. Sci., 45: 112.

Ogasawara, F.X., Lorenz, F.W. and Bobr, L.W. (1966) Distribution of spermatozoa in the oviduct and fertility in domestic birds. III. Intrauterine insemination of semen from low fecundity cocks. J. Reprod. Fert., 11: 33-41.

Okamura, F. and Nishiyama, H. (1978) The passage of spermatozoa through the vitelline membrane in the domestic fowl, Gallus gallus. Cell Tissue Res., 188: 497-508.

Oliphant, G. and Singhas, C.A. (1979) Iodination of rabbit sperm plasma membrane: relationship of specific surface proteins to epididymal function and sperm capacitation. Biol. Reprod., 21: 937-944.

Olsen, M.W. and Neher, B.H. (1948) The site of fertilization in the domestic fowl. J. Exp. Zool., 109: 355-366.

Olsen, M.W. (1966) Frequency of parthenogenesis in chicken eggs. J. Hered., 57: 23-25.

Olson, G.E. and Hamilton, D.W. (1978) Characterization of the surface glycoproteins of rat spermatozoa. Biol. Reprod., 19: 26-35.

Olson, G.E. and Danzo, B.J. (1981) Surface changes in rat spermatozoa during epididymal transit. Biol. Reprod., 24: 431-443.

Olson, G.E. (1984) Changes in the sperm surface during maturation in the epididymis. In Ultrastructure of Reproduction, pp. 97-109 (Eds. J. Van Blerkom and P.M. Motta). Martinus Nijhoff, The Hague.

O'Rand, M.G. (1986) Steps in the fertilization process: understanding and control. In The Molecular and Cellular Biology of Fertilization, pp. 383-393 (Ed. J.L. Hedrick). Plenum Press, New York.

O'Rand, M.G. and Fisher, S.J. (1987) Localization of the zona pellucida binding sites on rabbit spermatozoa and induction of the acrosome reaction by solubilized zonae. *Dev. Biol.*, 119: 551-559.

O'Rand, M.G. (1988) Sperm-egg recognition and barriers to interspecies fertilization. *Gamete Res.*, 19: 315-328.

Orgebin-Crist, M.-C. and Fournier-Delpech, S. (1982) Sperm-egg interaction: evidence for maturation changes during epididymal transit. *J. Androl.*, 3: 429-433.

Orlando, C., Casano, R., Forti, G., Barni, T., Vannelli, G.B., Balboni, G.C. and Serio, M. (1988) Immunologically reactive albumin-like protein in human testis and seminal plasma. *J. Reprod. Fert.*, 83: 687-692.

Overstreet, J.W. and Katz, D.F. (1977) Sperm transport and selection in the female tract. In Development in Mammals, Vol. 2, pp. 31-66 (Ed. M.H. Johnson). Elsevier, Amsterdam.

Overstreet, J.W. (1983) Transport of gametes in the reproductive tract of the female mammal. In Mechanism and Control of Animal Fertilization, pp. 499-543 (Ed. J.F. Hartmann). Academic Press, New York.

Overstreet, J.W., Clisham, P.R. and Boyers, S.P. (1989) Sperm transport and unexplained infertility. Symp. Unexplained Infert: Basic Clin. Aspects, Rome., pp. 285-296.

Overstreet, J.W. and Katz, D.F. (1990) Interaction between the female reproductive tract and spermatozoa. In Controls of Sperm Motility: Biological and Clinical Aspects, pp. 63-75 (Ed. C. Cagaon). CRC Press.

Pandya, I.J. and Cohen, J. (1985) The leukocytic reaction of the human cervix to spermatozoa. Fertil. Steril., 43: 417-421.

Parish, W.C., Caron-Brown, J.T. and Richards, C.B. (1967) The detection of antibodies to spermatozoa and to blood group antigens in cervical mucus. J. Reprod. Fert., 13: 469-483.

Parr, E.L. and Parr, M.B. (1985) Secretory immunoglobulin binding to bacteria in the mouse uterus after mating. J. Reprod. Immunol., 8: 71-82.

Parr, E.L. and Parr, M.B. (1988) Anti-bacterial IgA and IgG in mouse uterine luminal fluid, vaginal washings and serum. J. Reprod. Immunol., 13: 65-72.

Perry, M.M., Gilbert, A.B. and Evans, A.J. (1978) The structure of the germinal disc region of the hen's ovarian follicle during the rapid growth phase. J. Anat., 127: 379-392.

Phillips, D.M. and Mahler, S. (1977) Leucocyte emigration and migration in the vagina following mating in the rabbit. Anat. Rec., 189: 45-60.

Phillips, R.E. and Warren, D.C. (1937) Observations concerning the mechanics of ovulation in the fowl. J. Exp. Zool., 76: 117-136.

Price, R.J. and Boettcher, B. (1979) The presence of complement in human cervical mucus and its possible relevance to infertility in women with complement-dependent sperm immobilizing antibodies. Fertil. Steril. 32: 61-66.

Pycraft, W.P. (Ed.) (1931) The Standard Natural History from Amoeba to Man. Frederick Warine, London.

Racey, P.A. (1973) The viability of spermatozoa after prolonged storage by male and female European bats. Period. Biol., 75: 201-205.

Rankin, T.L., Tsuruta, K.J., Holland, M.K., Griswold, M.D. and Orgebin-Crist, M.-C. (1992a) Isolation, Immunolocalization, and sperm association of three proteins of 18, 25 and 29 kilodaltons secreted by the mouse epididymis. Biol. Reprod., 46: 747-766.

Rankin, T.L., Ong, D.E. and Orgebin-Crist, M.-C. (1992b) The 18 kDa mouse epididymal protein (MEP10) binds retinoic acid. Biol. Reprod., 46: 767-771.

Räsänen, M.L., Hovatta, O.L., Penttilä, I.M. and Agrawal, Y.P. (1992) Detection and quantitation of sperm-bound antibodies by flow cytometry of human semen. J. Androl., 13: 55-64.

Ravid, A., Sagiv, M., Bartoov, B., Eltes, F., Novogrodsky, A., Allalouf, D., Levinsky, H. and Singer, R. (1990) Separation of subpopulations of sperm with higher fertility potential from normal and pathological semen by peanut agglutinin. *Andrologia*, 22: 225-230.

Reviere, M. de (1975) Sperm transport and survival in male birds. In The Biology of Spermatozoa, pp. 10-16 (Eds. E. S. E. Hafez and C. G. Thibault). Karger, Basel.

Rifkin, J. M. and Olson, G. E. (1985) Characterization of maturation-dependent extrinsic proteins of the rat sperm surface. *J. Cell Biol.*, 100: 1582-1591.

Roldan, E. R. S., Vitullo, A. D., Merani, M. S. and Von Lawzewitsch, I. (1985) Cross fertilization in-vivo and in-vitro between three species of vesper mice, Calomys (Rodentia, Cricetidae). *J. Exp. Zool.*, 233: 433-442.

Roldan, E. R. S. and Yanagimachi, R. (1989) Cross-fertilization between syrian and chinese hamsters. *J. Exp. Zool.*, 250: 321-328.

Rothwell, B. and Tingari, M. D. (1973) The ultrastructure of the boundary tissue of the seminiferous tubule in the testis of the domestic fowl (Gallus domesticus). *J. Anat.*, 114: 321-328.

Russell, L.D., Peterson, R.N., Russell, T.A. and Hunt, W. (1983) Electrophoretic map of boar sperm plasma membrane polypeptides and localization and fractionation of specific polypeptide subclasses. Biol. Reprod., 28: 393-413.

Russell, L.D., Peterson, R.N., Hunt, W. and Strack, L.E. (1984) Post-testicular surface modifications and contributions of reproductive tract fluids to the surface polypeptide composition of boar spermatozoa. Biol. Reprod., 30: 959-978.

Russell, L.D., Montag, B., Hunt, W. and Peterson, R.N. (1985) Properties of boar sperm plasma membranes (PM): proteins released by washing and differential solubility in salts, detergents, and sensitivity to surface radiolabelling. Gamete Res., 11: 237-252.

Sacco, A.G., Subramanian, M.G. and Yurewicz, E.C. (1984) Association of sperm receptor activity with a purified pig zona antigen (PPZA). J. Reprod. Immunol., 6: 89-103.

Sacco, A.G., Yurewicz, E.C. and Subramanian, M.G. (1986) Carbohydrate influences the immunogenic and antigenic characteristics of ZP3 macromolecule (Mr 55000) of the pig zona pellucida. J. Reprod. Fert., 76: 575-586.

Saeki, Y., Tanabe, Y., Katsurogi, T. and Miyagi, M. (1963) Studies on the advance and distribution of cock sperm in the oviduct, with special reference to the tracing method with ^{32}P -labelled sperm. Bull. Nat. Inst. An. Indus, Chiba., 3: 91-98.

Schauer, R. (1985) Sialic acids and their role as biological masks. Trends Biochem. Sci., 10: 357-360.

Schindler, H., Ben-David, E., Hurwitz, S. and Kempenich, O. (1967) The relation of spermatozoa to the glandular tissue in the storage sites of the hen oviduct. Poult. Sci., 46: 1462-1471.

Schmell, E.D. and Gulyas, B.J. (1980) Ovoperoxidase activity in ionophore treated mouse eggs. II. Evidence for the enzyme's role in hardening the zona pellucida. Gamete Res., 3: 279-290.

Schuppin, G.T., Van Krey, H.P. and Denbow, D.M. (1984) Ultrastructural analyses of uterovaginal sperm storage glands in fertile and infertile turkey breeder hens. Poult. Sci., 63: 1872-1882.

Shalgi, R., Matityahu, A., Gaunt, S.J. and Jones, R. (1990) Identification of antigens on rat spermatozoa with a potential role in fertilization. *Mol. Reprod. Dev.*, 25: 286-296.

Shur, B.D. (1989) Galactosyltransferase as a recognition molecule during fertilization and development. In The Molecular Biology of Fertilization, pp. 37-70. Academic Press, New York.

Siller, W.G. (1956) A Sertoli tumor causing feminization in a brown leghorn capon. *J. Endocr.*, 74: 467-476.

Singh, J.P., Babcock, D.F. and Lardy, H.A. (1978) Increased calcium ion influx is a component of capacitation of spermatozoa. *Biochem. J.*, 172: 549-556.

Sinosich, M.J. and Saunders, D.M. (1987) Potential role of pregnancy associated plasma protein A in human reproduction. *J. Reprod. Immunol.*, 10: 55-65.

Smallcombe, A. and Tyler, K.R. (1980) Semen elicited accumulation of antibodies and leukocytes in the rabbit female tract. *Experientia*, 36: 88-90.

Smith, T.T., Koyanagi, F. and Yanagimachi, R. (1987)
Distribution and number of spermatozoa in the oviduct of
the golden hamster after natural mating and artificial
insemination. Biol. Reprod., 37: 225-234.

Smith, T.T., Koyanagi, F. and Yanagimachi, R. (1988)
Quantitative comparison of the passage of homologous and
heterologous spermatozoa through the uterotubal junction
of the golden hamster. Gamete Res., 19: 227-234.

Srivastava, A. and Olson, G.E. (1991) Glycoprotein
changes in the rat sperm plasma membrane during maturation
in the epididymis. Mol. Reprod. Dev., 29: 357-364.

Steele, M.G. and Wishart, G.J. (1992) Evidence for a
species-specific barrier to sperm transport within the
vagina of the chicken hen. Theriogenology, In-press.

Steklenev, E.P. and Kozikova, L.V. (1989) Peculiarities
of hybridization of domestic hen and guinea fowl and
structural-functional organization of hybrid chromosomes
as against initial species. Tsitologiya I Genetika, 23:
20-26.

Storey, B.T., Lee, M.A., Muller, C., Ward, C.R. and Wirtshafter, D.G. (1984) Binding of mouse spermatozoa to the zonae pellucidae of mouse eggs in cumulus: evidence that the acrosomes remain substantially intact. Biol. Reprod., 31: 1119-1128.

Stott, D.I. (1988) Immunoblotting and dot blotting. J. Immunol. Meth., 119: 153-187.

Suarez, S.S. (1987) Sperm transport and motility in the mouse oviduct: observations in-situ. Biol. Reprod., 36: 203-210.

Sullivan, R. and Robitaille, G. (1989) Heterogeneity of epididymal spermatozoa of the hamster. Gamete Res., 24: 229-236.

Symons, D.B.A. (1967) Reactions of spermatozoa with uterine and serum globulin determined by immunofluorescence. J. Reprod. Fert., 14: 163-165.

Takeda, A. (1974) The transport of spermatozoa in the vagina of the hen. Jap. J. Poult. Sci., 11: 45-54.

Tanaka, K. and Koga, O. (1971) Studies on the maintenance of fertilizing spermatozoa in the hen's oviduct. Jap. J. Zootech. Sci., 42: 237-249.

Taylor, N.J. (1982a) Equivalence of 'non-IgG binding' and 'acrosomeless' sperm populations from the female genital tract of the rabbit. J. Reprod. Fert., 64: 181-184.

Taylor, N.J. (1982b) Investigation of sperm-induced cervical leucocytosis by a double mating study in rabbits. J. Reprod. Fert., 66: 157-160.

Terada, T., Ashizawa, K. and Watanabe, M. (1981) Effects of pH on motility and metabolism of cock spermatozoa. Jap. Poult. Sci., 18: 40-44.

Tezon, J.G., Ramella, E., Gameo, M.S., Vazquez, M.H. and Blacquier, J.A. (1985) Immunochemical localization of secretory antigens in the human epididymis and their association with spermatozoa. Biol. Reprod., 32: 591-597.

Thibault, C. (1972) Physiology and Pathophysiology of the fallopian tube. Int. J. Fert., 17: 1-13.

Thibault, C. (1973) Sperm transport and storage in vertebrates. J. Reprod. Fert., Suppl. 18: 39-53.

Thurston, R.J. and Hess, R.A. (1987) Ultrastructure of spermatozoa from domesticated birds: comparative study of turkey, chicken and guinea fowl. Scan. Microsc., 1: 1829-1838.

Tingari, M.D. (1971) On the structure of the epididymal region and ductus deferens of the domestic fowl (Gallus domesticus). J. Anat., 109: 423-425.

Tingari, M.D. (1972) The fine structure of the epithelial lining of the excurrent duct system of the testis of the domestic fowl (Gallus domesticus). Q. J. Exp. Physiol., 57: 271-295.

Tingari, M.D. and Lake, P.E. (1972) Ultrastructural evidence for resorption of spermatozoa and testicular fluid in the excurrent ducts of the testis of the domestic fowl, Gallus domesticus. J. Reprod. Fert., 31: 373-381.

Tingari, M.D. and Lake, P.E. (1973) Ultrastructural studies on the uterovaginal sperm host glands of the domestic hen, Gallus domesticus. J. Reprod. Fert., 34: 423-431.

Tortora, G.J. and Anagnostakos, N.P. (1990) Principles of Anatomy and Physiology. Harper and Row, New York.

Toshimori, K., Araki, S. and Oura, C. (1988) Masking of sperm maturation antigen by sialic acid in the epididymis of the mouse. Histochemistry, 90: 195-200.

Toshimori, K., Araki, S., Oura, C. and Eddy, E.M. (1991)
Loss of sperm surface sialic acid induces phagocytosis: an
assay with a monoclonal antibody T21, which recognizes a
54 k sialoglycoprotein. Arch. Androl., 27: 79-86.

Trummel, D.E., Fulcher, K.D. and Cloud, J.G. (1992)
Subpopulations of rainbow trout spermatozoa within
individual males as detected by a sperm-specific
monoclonal antibody. Aquaculture, 100: 329.

Tyler, K.R. (1977) Histological changes in the cervix of
the rabbit after coitus. J. Reprod. Fert., 49: 341-345.

Underdown, B.J. and Schiff, J.M. (1986) Immunoglobulin A:
strategic defense initiative at the mucosal surface. Ann.
Rev. Immunol., 4: 389-417.

Van Drimmelen, G.C. (1946) 'Spermneests' in the oviduct of
the domestic hen. J. S. Afr. Vet. Med. Assoc., 17: 42-52.

Van Krey, H.P. (1964) Storage and transport of
spermatozoa within the oviduct of the domestic fowl.
Ph.D. Thesis, Univ. California, Davis.

Van Krey, H.P., Ogasawara, F.X. and Pangborn, J. (1967)
Light and electron microscopic studies of possible sperm
gland emptying mechanisms. Poult. Sci., 46: 69-78.

Van Krey, H. P., Balander, R. J. and Compton, M. M. (1981) Storage and evacuation of spermatozoa from the uterovaginal sperm host glands in domestic fowl. *Poult. Sci.*, 60: 871-877.

Van Krey, H. P., Schuppin, G. T., Denbow, D. M. and Hulet, R. M. (1987) Turkey breeder hen infertility associated with plasma cells in the uterovaginal sperm storage glands. *Theriogenology*, 27: 913-921.

Villaroya, S. and Scholler, R. (1986) Regional heterogeneity of human spermatozoa detected with monoclonal antibodies. *J. Reprod. Fert.*, 76: 435-447.

Voglmayr, J. K. (1987) Surface protein changes in ram spermatozoa during maturation and capacitation. In New Horizons in Sperm Cell Research, pp. 3-18 (Ed. H. Mohri). Jap. Sci. Soc. Press, Tokyo.

Waldman, R. H., Cruz, J. M. and Rowe, D. S. (1972) Intravaginal immunization of humans with Candida albicans. *J. Immunol.*, 109: 662-664.

Wallace, H. (1974) Chiasmata have no effect on fertility. *Heredity*, 33: 423-429.

Warren, D.C. and Scott, H.M. (1934) Ovulation in the domestic hen. Science, 80: 461-462.

Wassarman, P.M. (1992) Mouse gamete adhesion molecules. Biol. Reprod., 46: 186-191.

Watanabe, M. (1964) The hybridization of the white leghorn male with the Japanese green pheasant female by insemination. Proc. 5th Int. Congr. Anim. Reprod. Artif. Insem., Trento., 3: 481-483.

Watanabe, M. (1967) Studies on deep freezing preservation of chicken semen. J. Fac. Fish. Anim. Husb., Hiroshima Univ., 20: 81-85.

Watanabe, M. and Terada, T. (1980) Studies on the deep freezing of fowl semen in pellet form. 9th Int. Congr. Anim. Reprod. Artif. Insem., Madrid., 5: 477-479.

Westfall, F.D. and Howarth, B. Jr. (1977) The effects of glycerol removal on cation concentration and morphology of chicken spermatozoa. Poult. Sci., 56: 1454-1456.

Westfall, F.D. and Howarth, B. Jr. (1978) The effect of glycerol and dilution on the release of glutamate oxaloacetic transaminase from chicken spermatozoa. Poult. Sci., 57: 1037-1041.

Wishart, G.J. (1982) Maintenance of ATP concentrations in and fertilizing ability of fowl and turkey spermatozoa in-vitro. J. Reprod. Fert., 66: 457-462.

Wishart, G.J. (1984a) Metabolism of fowl and turkey spermatozoa at low temperatures. J. Reprod. Fert., 70: 145-149.

Wishart, G.J. (1984b) Effects of lipid peroxide formation in fowl semen on sperm motility, ATP content and fertilizing ability. J. Reprod. Fert., 71: 113-118.

Wishart, G.J. and Dick, L.A. (1985) Effect of γ -radiation on fowl sperm function in-vitro and in-vivo. J. Reprod. Fert., 75: 617-622.

Wishart, G.J. and Palmer, F.H. (1985) Correlation of the fertilizing ability of semen from individual male fowls with sperm motility and ATP content. Br. Poult. Sci., 26: 375-380.

Wishart, G.J. and Ross, F.H. (1985) Characterization of a spectrophotometric technique for the estimation of fowl and turkey sperm motility. Gamete Res., 11: 169-178.

Wishart, G.J. and Palmer, F.H. (1986) The effect of cryopreservative at -196 °C on the viability of fowl and turkey spermatozoa assessed in-vitro. Anim. Reprod. Sci., 10: 317-324.

Wishart, G.J. (1987) Regulation of the length of the fertile period in the domestic fowl by numbers of oviducal spermatozoa, as reflected by those trapped in laid eggs. J. Reprod. Fert., 80: 493-498.

Wishart, G.J. and Ashizawa, K. (1987) Regulation of the motility of fowl spermatozoa by calcium and cAMP. J. Reprod. Fert., 80: 607-611.

Xu, K.P., Greve, T., Callesen, H. and Hyttel, P. (1987) Pregnancy resulting from in-vitro fertilization of bovine oocytes matured in-vitro. J. Reprod. Fert., 81: 501-504.

Yanagimachi, R. and Chang, M.C. (1963) Infiltration of leukocytes into the uterine lumen of the golden hamster during the oestrous cycle and following mating. J. Reprod. Fert., 5: 389-396.

Yanagimachi, R. (1964) The behaviour of hamster sperm to the hamster and mouse ova in-vitro. Proc. 5th Int. Congr. Anim. Reprod. Artif. Insem., Trento., 7: 292-294.

Yanagimachi, R. (1972) Penetration of guinea pig spermatozoa into hamster eggs in-vitro. J. Reprod. Fert., 28: 477-480.

Yanagimachi, R., Noda, Y.D., Fujimoto, M. and Nicolson, G.L. (1972) The distribution of negative surface charges on mammalian spermatozoa. Am. J. Anat., 138: 497-520.

Yanagimachi, R. (1973) Behavior and functions of the structural elements of the mammalian sperm head in fertilization. In The Regulation of Mammalian Reproduction, pp. 215-230 (Eds. S.J. Segal, R. Crozier, P.A. Corfman and P.G. Condcliffe). C.E. Thomas, Illinois.

Yanagimachi, R., Yanagimachi, H. and Rogers, B.J. (1976) The use of zona-free animal ova as a test system for assessment of the fertilizing capacity of human spermatozoa. Biol. Reprod., 15: 471-476.

Yanagimachi, R. (1977) Specificity of sperm-egg interaction. In Immunology of Gametes, pp. 255-289 (Eds. M. Edidin and M.H. Johnson). Cambridge University Press, Cambridge.

Yanagimachi, R. (1981) Mechanisms of fertilization in mammals. In Fertilization and Embryonic Development In-vitro, pp. 81-182 (Eds. L. Mastroianni and J.D. Biggers). Plenum Press, New York.

Young, L.G., Gould, K.G. and Hinton, B.T. (1986) Lectin binding sites on the plasma membrane of epididymal and ejaculated chimpanzee sperm. *Gamete Res.*, 14: 75-87.

Zaleski, M.B., Dubiski, S., Niles, E.G. and Cunningham, R.K. (1983) Immunogenetics. Pitman, Massachussets.

Zar, J.H. (1984) Biostatistical Analysis. Prentice-Hall, New Jersey.

Zavaleta, D. and Ogasawara, F.X. (1987) A review of the mechanism of the release of spermatozoa from the storage tubules in the fowl and turkey oviduct. *Wlds. Poult. Sci. J.*, 43: 132-139.

Zinaman, M., Drobnis, E.Z., Morales, P., Brazil, C., Kiel, M., Cross, N.L., Hanson, F.W. and Overstreet, J.W. (1989) The physiology of sperm recovered from the human cervix: acrosomal status and response to inducers of the acrosome reaction. *Biol. Reprod.*, 41: 790-797.

APPENDICES

Appendix 1. Table of Lectin Specificities

| lectin | carbohydrate group affinities |
|------------------------------------|-----------------------------------------------------------------------------------------------------------------------------------------|
| <u>Triticum vulgaris</u> (WGA) | $(D\text{-GlcNAc}\beta 1-4)_3 > (D\text{-GlcNAc}\beta 1-4)_2$ = $D\text{-GlcNAc}\beta 1-4(\text{Fuc } 1-6)\text{GlcNAc} >$ NeuNac |
| <u>Canavalia ensiformis</u> (ConA) | $\alpha\text{-D-Man} > \alpha\text{-D-Glc} > \alpha\text{-D-GlcNAc}$ |
| <u>Solanum tuberosum</u> | $(D\text{-GlcNAc}\beta 1-4)_3$ |
| <u>Tetragonolobus purpureas</u> | $\alpha\text{-L-Fuc}$ |
| <u>Limulus polyphemus</u> | NeuNac |
| <u>Erythrina corallodendron</u> | $\beta\text{-D-Gal}-(1-4)\text{-D-GlcNAc}$ |
| <u>Arachis hypogea</u> | $\beta\text{-D-Gal}-(1-3)\text{-D-GalNAc}$ |

Appendix 2. Reagents for SDS-PAGE and Western Blotting

a) Reagents for SDS-PAGE

I. Gel Reagents

Resolving Gel Acrylamide

| | |
|------------|---------|
| Acrylamide | 28.50 g |
|------------|---------|

| | |
|-------------------------------|--------|
| N,N'-methylene-bis-acrylamide | 1.50 g |
|-------------------------------|--------|

- dissolved in 100 ml of distilled water.

Stacking Gel Acrylamide

| | |
|------------|---------|
| Acrylamide | 29.25 g |
|------------|---------|

| | |
|-------------------------------|--------|
| N,N'-methylene-bis-acrylamide | 0.75 g |
|-------------------------------|--------|

- dissolved in 100 ml of distilled water.

Resolving Gel Buffer

| | |
|-----------|---------|
| tris base | 18.15 g |
|-----------|---------|

| | |
|-----|--------|
| SDS | 0.40 g |
|-----|--------|

- dissolved in 100 ml of distilled water

Stacking Gel Buffer

| | |
|-----------|--------|
| tris base | 5.90 g |
|-----------|--------|

| | |
|-----|--------|
| SDS | 0.40 g |
|-----|--------|

- dissolved in 100 ml of distilled water.

Resolving Gel (per 5-15% gradient gel)

| | 5% | 15% |
|------------------------------------------------------------------------------|------------|------------|
| | acrylamide | acrylamide |
| resolving gel acrylamide (ml) | 2.66 | 8.00 |
| resolving gel buffer (ml) | 4.00 | 4.00 |
| distilled water (ml) | 9.33 | 1.60 |
| ammonium persulphate (ul) | 100.00 | 43.33 |
| TEMED (N, N, N ^l , N ^l - tetraethylenediamine) (ul) | 6.60 | 6.60 |
| glycerol (ml) | 0.00 | 2.40 |

Stacking Gel (per gel)

| | |
|-------------------------|-----------|
| stacking gel acrylamide | 2.00 ml |
| stacking gel buffer | 3.00 ml |
| distilled water | 7.00 ml |
| ammonium persulphate | 100.00 ul |
| TEMED | 5.00 ul |

II. Boiling Mix

| | |
|------------------------------|---------|
| stacking gel buffer | 1.00 ml |
| 25% SDS | 0.80 ml |
| β -mercaptoethanol | 0.50 ml |
| glycerol | 1.00 ml |
| bromophenol blue (0.01% w/v) | 0.33 ml |

III. Tank Buffer

| | |
|-----------|--------|
| tris base | 15.8 g |
| SDS | 2.5 g |
| glycine | 10.0 g |

- dissolved in 2.5 l of distilled water.

b) Reagents for Western Blotting

I. Anode Buffer 1

| | |
|----------|---------|
| tris-HCl | 11.85 g |
|----------|---------|

| | |
|----------|----------|
| methanol | 50.00 ml |
|----------|----------|

- made up to 250 ml with distilled water
(final pH 10.4)

II. Anode Buffer 2

| | |
|----------|--------|
| tris-HCl | 0.99 g |
|----------|--------|

| | |
|----------|----------|
| methanol | 50.00 ml |
|----------|----------|

- made up to 250 ml with distilled water
(final pH 10.4)

III. Cathode Buffer

| | |
|----------|--------|
| tris-HCl | 0.79 g |
|----------|--------|

| | |
|--------------------|--------|
| amino-caproic acid | 1.31 g |
|--------------------|--------|

| | |
|----------|----------|
| methanol | 50.00 ml |
|----------|----------|

- made up to 250 ml with distilled water
(final pH 9.4)

Appendix 3. Phastsystem Reagents and Automated Silver
Staining Protocol

a) Phastsystem Reagents

I. Boiling Mix

| | |
|------------------------------------------------------|------|
| tris-HCl (1.6% w/v) | 1 ml |
| EDTA (ethylenediaminetetraacetic acid) (0.3% w/v) | 1 ml |
| SDS (25% w/v) | 1 ml |
| β -mercaptoethanol (50% v/v) | 1 ml |
| bromophenol blue (0.1% w/v) | 1 ml |
| distilled water | 5 ml |

b) Phastsystem Automated Silver Staining Protocol

| <u>Step</u> <u>Number</u> | <u>Solution</u> | <u>Time of</u> <u>Incubation</u> | <u>Incubation</u> <u>Temperature</u> |
|------------------------------|----------------------------------------------------------|-------------------------------------|-----------------------------------------|
| 1 | 50% ethanol, 10% acetic acid (v/v) | 2 min | 50 °C |
| 2 | 10% ethanol, 5% acetic acid (v/v) | 6 min | 50 °C |
| 3 | 8.3% gluteraldehyde (v/v) | 6 min | 50 °C |
| 4 | 10% ethanol, 5% acetic acid (v/v) | 8 min | 50 °C |
| 5 | distilled water | 4 min | 50 °C |
| 6 | 0.5% silver nitrate (w/v) | 10 min | 40 °C |
| 7 | distilled water | 1 min | 30 °C |
| 8 | 0.03% formaldehyde (v/v), 2.5% sodium carbonate (w/v) | 6 min | 30 °C |
| 9 | 5% acetic acid (v/v) | 2 min | 50 °C |
| 10 | 10% acetic acid, 5% glycerol (v/v) | 3 min | 50 °C |

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Appendix 4. Published Papers

1. Wishart, G.J. and Steele, M.G. (1990) The influence of surface characteristics on sperm function in the reproductive tract. In Control of Fertility in Domestic Poultry, pp. 101-112 (Ed. J.-P. Brillard) INRA, Paris.
2. Steele, M.G. and Wishart, G.J. (1991) A vaginal sperm recognition mechanism in domestic fowl hens recognises sperm proteins. J. Reprod. Fert., Abstract Series No. 7:
3. Steele, M.G. and Wishart, G.J. (1992) Characterisation of a sperm recognition mechanism impeding sperm transport through the vagina of the chicken. Proc. 12th Int. Congr. Anim. Reprod., pp. 1593-1595.
4. Wishart, G.J., Staines, H.J. and Steele, M.G. (1993) A method for predicting impending infertility in naturally-mated flocks and demonstration of gross variation in sperm transport efficiency. Proc. XIX Wld's Poultry Congr., pp. 631-634.